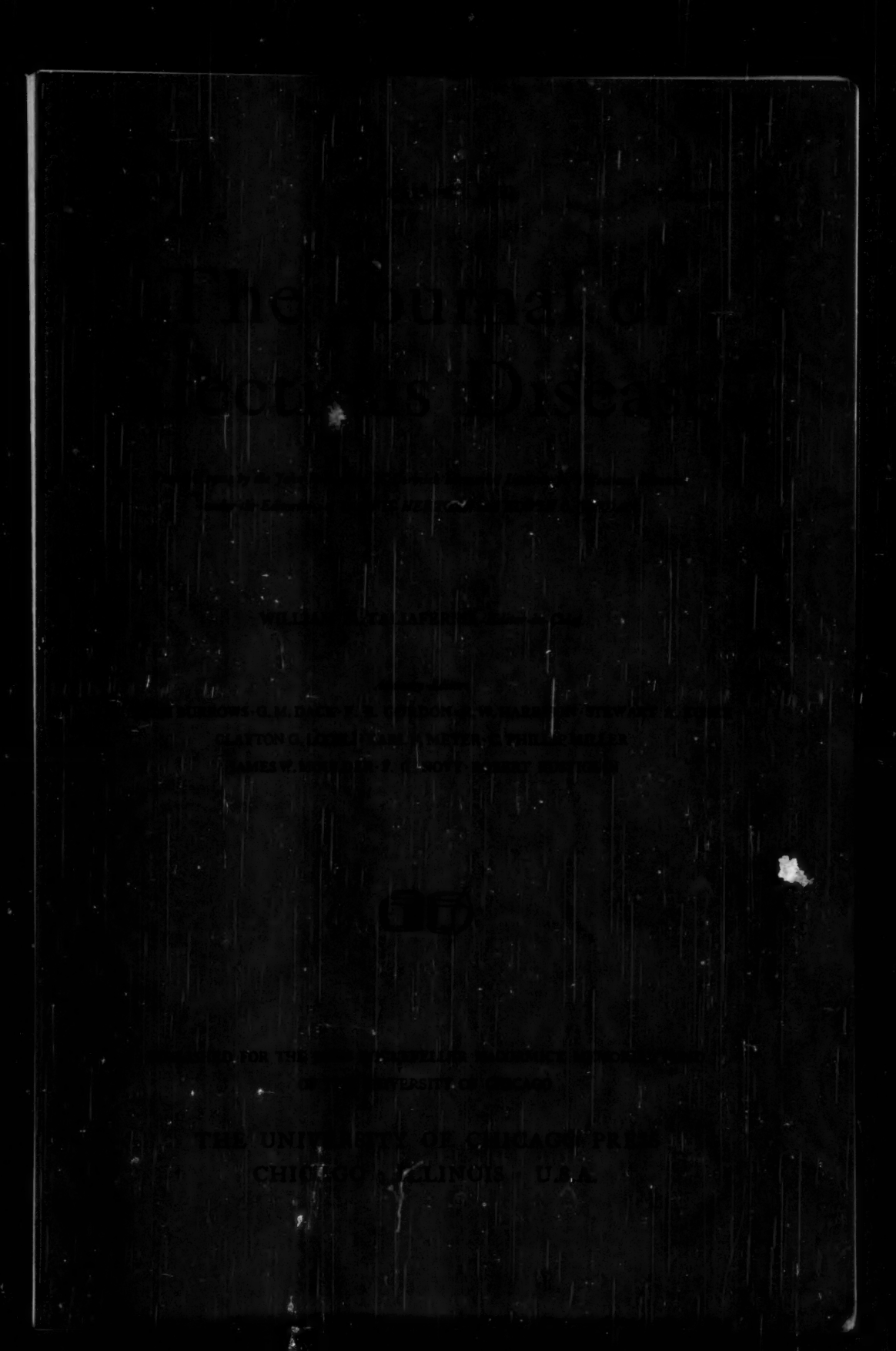

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THE ACTION OF DRUG COMBINATIONS ON *PLASMODIUM LOPHURAE* IN THE CHICK

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While the procedure of combining drugs has been used in the treatment of human malaria, particularly since the introduction of pamaquine as a gametocidal agent in 1926, it has not generally been based on quantitative data obtained in the laboratory. The testing procedure devised by Roehl[1] showed that pamaquine and quinine in combination markedly delayed the appearance of parasites as compared to quinine alone. This enhanced action of the combination was observed clinically by Muehlens,[2] Sinton and Bird,[3] Sinton, Smith, and Pottiger,[4] and Warthin et al.[5]

In this report quantitative data on the joint effect of quinine and quinacrine, quinacrine and pamaquine, quinine and pamaquine, and quinine and pentaquine will be presented. The results were analyzed by statistical treatment of the dose-response relationships.

MATERIALS AND METHODS

The procedure for determining antimalarial activity is similar to that described in a previous paper.[6] Briefly it is as follows: Young white Leghorn cockerels, 8 to 9 days old and weighing between 50 and 60 g, were inoculated with a suspension of red blood cells in 0.2 cc Ringer's solution of which 50×10^6 were parasitized with *Plasmodium lophurae*. For 5 days beginning 12 hours after inoculation, the chicks were given a drug-diet to be tested. Blood smears were made on the 3rd, 4th, and 5th days. Parasite counts were made from all smears to study the course of infection and from the 4th day to assay antimalarial activity.

For investigating the joint effect of two drugs, 10 to 13 groups of infected chicks of the same stock, 8 in each group, were employed in any one experiment. One control group was not treated. Of the remainder, 3 or 4 groups were fed varying amounts of each of the two drugs while 3 or 4 groups were fed the same minimal amount of one of the drugs and varying amounts of the other drug within a range around the 50% suppressive level. The actual amounts depended upon results previously obtained for a single drug. Combination tests were repeated at least twice to verify the relationship found.

The 50% suppressive dose and its standard error were calculated for the individual drugs by the log-dose-probit linear relationship.[7] The 50% suppressive dose for a combination of two drugs was also obtained from the dose-response curve, but no standard error could be obtained since the joint action of any combination may be either synergistic or independent.[8]

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3. Sinton, G. A. and Bird, W. 1928, Indian J. M. Research, Part IX, **16**: 159.
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8. Finney, D. J. 1947, Probit Analysis. Cambridge, Cambridge University Press, p. 122.

TABLE 1.—*The suppressive effect of quinine and quinacrine alone and together against* P. lophurae *in 12 groups of chickens as compared to a control group.*

Quinine		Quinacrine		Quinine and quinacrine			
Grams percent in diet	Percent suppression	Grams percent in diet	Percent suppression	Grams percent in diet	Percent suppression	50% antimalarial equivalent of	
				Qn Qc			Quinine + Quinacrine
0.02	15.0	0.01	17.5	0.02 +0.005	22.5	Range of grams percent in diet	0.48–0.71 +0.45–1.00
0.03	32.5	0.02	45.0	0.02 +0.010	40.0		
0.04	62.5	0.03	70.0	0.02 +0.020	65.0		
0.06	90.0	0.04	70.0	0.02 +0.030	80.0		
SD_{50} 0.035	50.0	0.021	50.0	0.02 +0.013	50.0		0.57 + 0.62
S.E. ±0.007		±0.008					Sum = 1.19

Qn = quinine, Qc = quinacrine, SD_{50} ± S.E. = 50% suppressive dose ± standard error.

The joint effect of antimalarials was defined in terms of equivalent doses.[9] The 50% suppression of parasitemia was taken as a standard measure of the antimalarial activity of drugs, singly or in combination. The 50% suppressive dose of an individual drug was arbitrarily considered to be equal to the value of one. Amounts of drugs in combination which produced a 50% suppression of parasitemia are expressed as fractions of one equivalent so defined. When one-half equivalent amounts to two drugs or three-quarters of one and one-quarter of the other, making a total of one equivalent, produce a 50% malarial suppression as compared to the control group, the joint effect is considered additive, i.e., equal to the summation of response by the two drugs. When larger amounts of two drugs than one equivalent are needed for 50% suppression, the joint effect is less than a summation of response. On the other hand, when smaller amounts of two drugs are needed, their joint effect may be considered synergistic. A graphical presentation of the combined antimalarial effects of the drugs used will be given in the following section.

9. Loewe, S. and Muischnek, F. 1926, Arch. f. exper. Path. u. Pharmakol. **114**: 313.

RESULTS AND DISCUSSION

Data on quinine and quinacrine, quinacrine and pamaquine, quinine and pamaquine, quinine and pentaquine alone and in combination are presented in tables 1, 2, 3, and 4, respectively. Ranges of the equivalent antimalarial values in the last column in each table were calculated from the two values of SD_{50} (50% suppressive dose) ± S.E. for the individual drugs. As may be seen in the 4 tables, the sum of the average equivalent values for the drugs tested were: Qn + Qc = 1.19, Qc + Pm = 1.13, Qn + Pn = 0.64, and Qn + Pt = 0.95. Thus, a combination of quinine and quinacrine or quinacrine and pamaquine necessitated more than one equivalent to produce a 50% suppression of parasitemia, whereas a combination of quinine and pamaquine or of quinine and pentaquine required less than one equiv-

TABLE 2.—*The suppressive effect of quinacrine and pamaquine alone and together against* P. lophurae *in 10 groups of chickens as compared to a control group.*

Quinacrine		Pamaquine		Quinacrine and pamaquine			
Grams percent in diet	Percent suppression	Grams percent in diet	Percent suppression	Grams percent in diet	Percent suppression	50% antimalarial equivalent of	
				Qc Pm			Quinacrine + Pamaquine
0.005	7.1	0.0005	7.1	0.005 +0.0005	7.1	Range of grams percent in diet	0.69–0.87 +0.24–0.71
0.010	31.2	0.0010	23.7	0.01 +0.0005	23.7		
0.020	31.6	0.0020	70.0	0.02 +0.0005	50.0		
0.040	70.0						
SD_{50} 0.026	50.0	0.0014	50.0	0.02 +0.0005	50.0		0.77 + 0.36
S.E. ±0.003		±0.0007					Sum = 1.13

Pm = pamaquine. Other abbreviations as in table 1.

TABLE 3.—*The suppressive effect of quinine and pamaquine alone and together against* P. lophurae *in 12 groups of chickens as compared to a control group.*

Quinine		Pamaquine		Quinine and pamaquine				
Grams percent in diet	Percent suppression	Grams percent in diet	Percent suppression	Grams percent in diet Qn	Grams percent in diet Pm	Percent suppression	50% antimalarial equivalent of	Quinine + Pamaquine
0.02	18.2	0.0005	4.6	0.005	+0.0005	27.3	Range of grams percent in diet	0.21–0.41 + 0.29–0.55
0.03	45.5	0.0010	18.2	0.01	+0.0005	63.7		
0.04	54.6	0.0015	68.2	0.02	+0.0005	68.2		
0.06	77.3	0.0020	81.9	0.03	+0.0005	81.8		
SD_{50} 0.035	50.0	0.0013	50.0	0.0098	+0.0005	50.0		0.28 + 0.38
S.E. ±0.011		±0.0004						Sum = 0.64

Abbreviations as in tables 1 and 2.

alent to produce a similar effect.

A clearer picture of the antimalarial effect of these drugs may be seen from the graphical presentation of the data in figure 1, in which the equivalent quantities of the drugs tested were plotted along two axes. The straight diagonal line connecting one equivalent dose of two drugs represents the 50% suppression line for a combination of two drugs which produce a summation in response. Any point or curve between the line and the origin indicates a synergistic action of the two drugs, and the farther away from this line the point is, the greater is the synergism. The region beyond this line indicates a joint effect less than the summation of response of the individual drugs. The area bounded by the range of equivalent doses for each combination of drugs is the approximate limit of scatter due to biological variation, so that our results are expected to lie within the present design of the assay procedure. The area of scatter, of course, may be reduced by increasing the number of birds used in the test. It appears evident, however, from the present data that combinations of quinine and quinacrine or of quinacrine and pamaquine act additively and that those of quinine and pamaquine and possibly those of quinine and pentaquine act synergistically in suppressing *P. lophurae* infection in the chick.

TABLE 4.—*The suppressive effect of quinine and pentaquine alone and together against* P. lophurae *in 12 groups of chickens as compared to a control group.*

Quinine		Pentaquine		Quinine and pentaquine				
Grams percent in diet	Percent suppression	Grams percent in diet	Percent suppression	Grams percent in diet Qn	Grams percent in diet Pt	Percent supression	50% antimalarial equivalent of	Quinine + Pentaquine
0.01	25.1	0.0000625	25.1	0.01	+0.0000625		Range of grams percent in diet	0.33–1.00 + 0.23–1.25
0.02	42.7	0.000125	47.1	0.02	+0.0000625			
0.03	42.7	0.000250	73.5	0.03	+0.0000625			
0.04	64.8	0.000500	86.7	0.04	+0.0000625			
SD_{50} 0.040	50.0	0.000140	50.0	0.020	+0.0000625			0.50 + 0.45
S.E. ±0.020		±0.00009						Sum = 0.95

Pt = pentaquine. Other abbreviations as in tables 1, 2, and 3.

The findings are in agreement with the observations of Chen and Geiling[10] on the acute lethal toxicity of these combinations in mice. The synergistic toxic action of the combination of quinine and pentaquine is less than that of quinine and pamaquine. The results are also in harmony with the clinical

10. Chen, G. M. and Geiling, E. M. K. 1946, Federation Proc. **5**: 170.

observations of Alving,[11] Trager et al,[12] and of others already mentioned on the curative effect of these combinations of drugs in vivax malaria. A combination of quinine and pamaquine or of quinine and pentaquine prevents relapse while quinacrine and pamaquine together are not more effective than quinacrine alone. On the other hand, Bratton[13] did not find a summation of response in the suppression of lophurae infection of the duck following the continuous intravenous injection of such combinations of antimalarials. He suggested a different principal mechanism of drug action against *P. lophurae* in the duck.

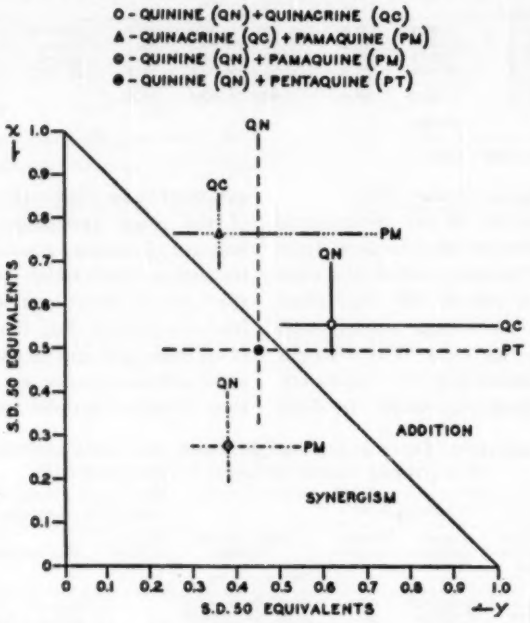

FIG. 1.—Equivalent quantities of various combinations of drugs are plotted along two axes. The diagonal line connecting one equivalent dose of each drug at the corresponding axis is a 50% suppression line for a combination of two drugs which produce an exact summation in response. Points above this line indicate additive effects in which the summation is less than one and are progressively less the farther they are above the line. Points below this line indicate additive effects greater than one, i.e., a synergism, and are progressively greater the farther they are below the line. The range of equivalent doses which is given for each drug and combination of drugs indicates the approximate limit of scatter variation. Qn = quinine, Qc = quinacrine, Pm = pamaquine, and Pt = pentaquine.

The recent report of Walker and Richardson is of interest in that the curative action of pamaquine or pentaquine and the naphthoquinones is potentiated by the drug-diet regime in cathemerium infection of the duck, while the suppressive action of these drug combinations is not potentiated.[14] These authors have also found that in

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12. Trager, W., Bang, F. B. and Hairston, W. G. 1947, Am. J. Hyg. **45**: 43.
13. Bratton, A. C., Jr. 1945, J. Pharmacol. & Exper. Therap. **85**: 103.
14. Walker, H. A. and Richardson, A. P. 1948, J. Nat. Malaria Soc. **7**: 4.

this avian malaria the ratios of the curative dose to the suppressive dose for quinacrine and pamaquine are approximately the same.[15]

SUMMARY

The joint effects of quinine and quinacrine, quinacrine and pamaquine, quinine and pamaquine, and quinine and pentaquine have been investigated by the drug-diet regime in *Plasmodium lophurae* infection of the chick. The data were analyzed in terms of equivalent doses of each antimalarial in producing a 50% suppression of parasitemia.

Quinine and quinacrine as well as quinacrine and pamaquine were found to be additive, whereas quinine and pamaquine and possibly quinine and pentaquine were synergistic in their joint suppressive effects.

15. Walker, H. A., Stauber, L. A. and Richardson, A. P. 1946, Federation Proc. **5**: 170.

COMPARISON OF THE ACTIONS OF CHLORAMPHENICOL (CHLOROMYCETIN*) AND PENICILLIN G AGAINST RELAPSING FEVER IN MICE†

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Spirochetes are included in the spectrum of pathogenic agents susceptible in vitro to the action of chloramphenicol (Chloromycetin).[1] In view of the limited number of therapeutic agents available for the treatment of relapsing fever, the discovery of antispirochetal action of a new drug is of interest. This is particularly true when the new agent is suitable for oral as well as parenteral administration in human therapeutics.[2–7] The present investigation, demonstrating a therapeutic action of chloramphenicol against experimental relapsing fever infections in mice and evaluating certain aspects of this action with reference to that of penicillin G, provides additional encouragement for exploration of chloramphenicol in the treatment of relapsing fever of man.

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* Parke, Davis & Company's trademark for chloramphenicol.

† Preliminary note: 1949, Fed. Proc. **8**: 338.

‡ Kindly supplied by Dr. Q. M. Geiman, Harvard Medical School.

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MATERIALS AND METHODS

Female white mice (Harlan Farms) weighing from 12 to 20 g were used both for routine passages of the test organism and for the chemotherapeutic studies.

The strain of *Borellia novyi*‡ employed had been maintained for over a year in mice, in which it is pathogenic and, under optimal conditions, produces reasonably uniform infections.[8] Presumably the organism is capable of causing relapsing fever in man.

Maintenance of the strain, procedures for producing test infections, and criteria for determining the effects of therapy were similar to those of Richardson et al.[8] For transmission of infections, blood was taken into heparinized saline solution by cardiac puncture from infected animals under ether anesthesia. The strain was maintained by intraperitoneal injection into serial groups of mice at 24- to 72-hour intervals. For chemotherapeutic studies, spirochetes were obtained from donors infected 24 to 48 hours previously, and test animals were inoculated intraperitoneally with 1×10^{10} or 5×10^{10} organisms per kg of body weight. Enumeration of organisms in the inoculums was based on erythrocyte counts made with a hemocytometer and on the ratio of spirochetes to erythrocytes observed in stained blood smears. Smears used in evaluating the inoculums or the experimental spirochetemia levels (tail blood) were stained with MacNeal's tetrachrome solution. By use of a modification of methods[9,10] for determining the degree of para-

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sitemia in malarial infections, sufficient organisms and erythrocytes were counted so that ordinarily the probable error in evaluating the smears did not exceed 15%. Measurement of spirochetemia relative to erythrocytes suffers from the limitation that anemia occurs during the infection.[9] However, since the anemia depends on the infection, it would be expected to occur in similar degrees at equivalent chemotherapeutic levels of various agents and would hardly be expected to bias comparison of such agents.

Crystalline sodium penicillin G (1667 International Units per mg)[11] served as a suitable reference drug for the new antibiotic because of its established use as an antispirochetal agent, its activity against experimental relapsing fever itself,[9,12,13] and because, like chloramphenicol, it is a chemically homogeneous antibiotic. The pure, natural, crystalline chloramphenicol[14,15] used has been defined chemically as D(-)threo-2-dichloroacetamido-1-p-nitrophenyl-1,3-propanediol.[16]

The drugs were administered to infected animals either in a diet of mash* or by intraperitoneal injection. In treatment by the diet method the animals were kept in individual cages with food cups† designed to minimize wastage of the drug diet. Doses are expressed in mg of drug consumed per kg of mean body weight (average of initial and final weights) per average day of a specified period. For other methods of administering drugs, freshly prepared solutions in physiological saline were adjusted to provide the desired amount per kg of body weight in a volume of 0.2 to 0.4 cc per mouse. Doses of penicillin G are expressed as the amount of sodium salt of the drug.

The drugs were compared simultaneously in terms of their abilities to suppress acute spirochetemias and to protect animals against the lethal effects of the infection. Each experiment included a group of 10 to 13 untreated mice and similar groups treated with several dose levels of chloramphenicol and of penicillin G, respectively. Mice within an experiment were infected from a common pool of spirochetes.

Studies on plasma levels of chloramphenicol employed uninfected mice of mixed strains and sexes, weighing from 16 to 24 g. Following administration of the drug to a pool of animals, groups of 10 were sacrificed at various intervals. Blood was taken by cardiac puncture into needles and syringes moistened with a saturated solution of heparin, and was pooled for each interval. Plasma was separated from the cells by centrifugation and used for parallel determinations of microbiologically active chloramphenicol by assay with *Shigella sonnei*[17] and of total nitro compounds, including chloramphenicol, by colorimetric assay.[18] Experiments were done with single, 50 mg/kg doses, intraperitoneally, subcutaneously, and by stomach tube, and by administration in the diet.

RESULTS

Plasma levels of chloramphenicol in normal mice.—Results of the single dose experiment are shown in table 1. Chloramphenicol was absorbed rapidly into the bloodstream of mice following either parenteral or oral administration. Peak concentrations occurred in the plasma within 15 or 30 minutes. The amount of active drug declined to a low level within one to a few hours after a single dose, but measurable levels persisted longer with oral than with parenteral administration. The amounts of active drug were consistently less than the amounts of total nitro compounds. This indicates that a considerable portion of the chloramphenicol administered to mice is converted to less active nitro derivatives, which is in accord with the degra-

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15. Bartz, Q. R. 1948, J. Biol. Chem. **172**: 445–450.
16. Rebstock, M. C., Crooks, H. M., Jr., Controulis, J. and Bartz, Q. R. 1949, J. Am. Chem. Soc. **71**: 2458–2462.

* Percentage composition: whole cornmeal, 33; ground whole wheat, 31; linseed oil meal (old process), 7; alfalfa meal, 2; whole milk powder, 21; dried hog liver, 3; brewers yeast (Strain G), 2; sodium chloride, 0.5; calcium carbonate, 0.5. Prepared in Vitamin Section of these Laboratories.

† Designed in the Dept. of Pharmacology, University of Michigan.

17. Joslyn, D. A. and Galbraith, M. 1947, J. Bact. **54**: 26.
18. Glazko, A. J., Wolf, L. M. and Dill, W. A. 1949, Arch. Biochem. **23**: 411–418.

dation of the drug in various other species, including man.[19]

Thirty mice on a diet of mash containing 0.0312% chloramphenicol with an average drug intake of 67 mg/kg/day were sacrificed in groups of 10 at 6, 12, and 24 hours after the beginning of the drug diet. These samples had less than 1 μg of active drug per cc of plasma but approximately 2 μg of total nitro compounds, expressed in each case as chloramphenicol equivalents.

spirochetes died within 15 days. All but an occasional animal died with 2.5×10^{10}, and all died within 7 days with 5×10^{10} spirochetes/kg.

Effects of treatment by the drug-diet method.—Groups of 10 mice each were placed on diets containing various percentages of either drug (0.0004–0.0240% penicillin G and 0.0040–0.0632% chloramphenicol) or none (infection controls). Eighteen to 24 hours later they were infected with 1×10^{10} spiro-

TABLE 1.—*Plasma levels of chloramphenicol activity and of total nitro compounds in mice following a single dose of 50 mg of chloramphenicol per kg of body weight.*

Time after administration (hours)	μg of chloramphenicol equivalents per cc					
	Microbiological activity*			Total nitro compounds (colorimetric)*		
	Intra-peritoneal	Subcutaneous	Oral	Intra-peritoneal	Subcutaneous	Oral
1/4	16	21	17	34	33	26
1/2	12	19	14	24	34	28
		16	15		29	26
			16			28
1	<1	4	13	15	13	23
2	<1	<1	3	5	5	14
4	<1	<1	2	1	2	6
8		<1	<3		1	2
16		<1			0	

* Each pair of microbiological and colorimetric determinations, at specified interval and route, were made from the pooled plasma of a separate group of 10 animals.

Observations on untreated infections.—The general host-parasite relationships in acute *B. novyi* infections of the mouse have been described by Richardson et al.[8] The methods of these workers were applied without important modification and resulted in confirmation of their findings. It is, therefore, necessary to describe only those aspects of the infections involved in evaluating the effects of therapy. Following inoculation with 1×10^{10} spirochetes/kg, the blood infection in a majority of the animals reached a peak after approximately 48 hours, usually amounting to between 6000 and 10,000 spirochetes per 10,000 erythrocytes. Approximately half of the animals inoculated with this number of

19. Glazko, A. J., Wolf, L. M., Dill, W. A. and Bratton, A. C., Jr. 1949, J. Pharmacol. & Exper. Therap. **96:** 445–459.

chetes/kg, and blood smears were made 46 to 48 hours after infection. The drug diet was continued for two days beyond the time of taking smears with the hope of obtaining better information on protection against death. In one experiment involving 90 mice, feeding data were collected on individual animals. Wide fluctuations occurred in the degree of spirochete suppression among individual animals having comparable drug intakes.[8] Individual intakes were not determined for two other experiments, and in the final analysis mean drug intake (total 5-day period), mean spirochetemia, and proportion of survivals in groups of animals at respective diet percentages and drugs constituted the variates studied.

Results of the three experiments are given in figure 1 and its legend. Certain

features of the data are apparent by inspection. Real degrees of suppression of blood parasites and protection against deaths occurred with either drug, but generally more chloramphenicol than penicillin G was required for a given effect. Substantial parallelism between the drugs in their suppression of blood spirochetes admits the possibility of similar qualities of over-all action on the difference between the drugs in access to vital aspects of tissue infection.

Variability in vital protection, particularly with penicillin G, together with the uncertainty introduced by a high proportion of survivals in controls, discouraged attempts to analyze further the protection data of these first experiments. However, the more systematic spirochetemia data have been examined

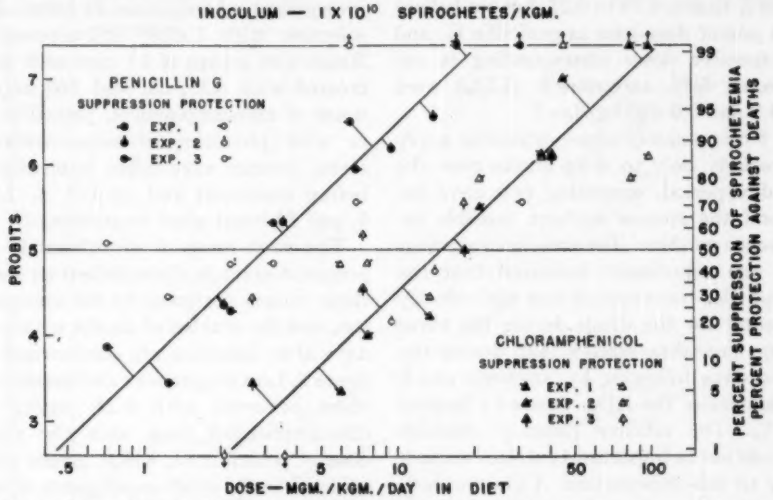

FIG. 1. Comparison of chloramphenicol and penicillin G, in the diet, in suppression of spirochetemia and in protection against deaths.

Drug administration was begun 18 to 24 hours before infection, and blood smears for determining spirochetemia were taken at 46 to 48 hours after infection. The drug was continued for two days beyond taking smears (see text), and the average daily intake for each group of animals determined for the total 5-day period. Deaths were observed for 15 days after infection.

The difference between treated and control (6700, 5843, and 8346 spirochetes per 10,000 erythrocytes in the respective experiments) spirochetemias, expressed as a percentage of the latter, is termed suppression. The percent protection against death is corrected[20] according to the estimate of independent natural resistance to deaths provided by the controls (3/10, 5/10, and 3/10 survival in the respective experiments).

parasites. Nevertheless, the suggestion is strong that for protection against death, requirements of individual groups of animals for penicillin G are less predictable than are requirements for chloramphenicol. This contrast between similarity in suppression of spirochetemia and probable dissimilarity in protection against death suggests a statistically. By inspection there is substantial homogeneity among the three experiments, and linearity in the log dose-probit effect relationship. A modification of standard probit analysis*

* Since the empirical probits arose from inferred proportions rather than by enumeration from simple spirochete samples of definite numbers, relative instead of absolute weights were

sustained a lack of significant difference between the two drugs in log dose-probit suppression slope or in variances. Hence, the common average slope of the regression lines, shown in the figure, and a simple, over-all relative potency computation indicate the essential comparative features of the two drugs in suppression of spirochetemia. Chloramphenicol was computed to be 0.20 (95% limits, 0.16 to 0.25; but see below) as potent dose-wise as penicillin G, and respective doses corresponding to expected 50% suppression (ED_{50}) were 14.9 and 3.0 mg/kg/day.†

Unfortunately these estimates apply precisely only to drug intake over the 5-day period, extending two days beyond the smears without possible influence on them. The detailed weighings in one experiment indicated that the drug diet consumption was equivalently greater for the drugs during the three days preceding smears than during the two days following by amounts which might raise the ED_{50} values by around 20%. The relative potency estimate would not be importantly altered according to this information. A graphic estimate based on diet percentage, and subject to undetermined differential influences on eating of the drugs, yielded a potency ratio of 0.15.

Effects of single intraperitoneal doses of drugs.—Information on the time required for various well-defined dose levels of drugs to suppress the spirochetemia, as well as on the degree and duration of effect, was obtained by single interperitoneal treatment 24 hours after infection with 1×10^{10} spirochetes/kg. Respective groups of 10 mice each were treated with 6.25, 25, and 100 mg/kg doses of chloramphenicol, penicillin G, or with physiological saline solution alone. Smears were taken immediately before treatment and at 1/2, 1, 2, 4, 8, and 24 hours after treatment.

The mean levels of spirochetemia for groups of animals, the standard errors of those means pertinent to the comparison, and the number of deaths within 15 days after infection are summarized in figure 2. Less suppressive and protective effect occurred with 6.25 mg/kg of chloramphenicol than with the same dose of penicillin G, which agrees with evidence in the other experiments of less activity, particularly by small doses of chloramphenicol. Otherwise, the actions of corresponding dose levels of the drugs were similar. Spirochete suppression occurred within one hour and became increasingly marked up to 2 or 4 hours after treatment. The degree and duration of suppressive effect were related to the dosage. Protection against death occurred with all dose levels with the possible exception of 6.25 mg/kg of chloramphenicol. Like penicillin G,[24] the blood level of chloramphenicol in normal mice (table 1) reaches a peak within 30 minutes after parenteral administration and declines to very low levels during

used in computing the regressions; and the resulting relative variances were converted to empirical variances by means of the computational expression for the weighted mean square deviation[21,22] that ordinarily gives a Chi-square divided by its degrees of freedom when weighting is absolute.[20,23]

† The combination of experimental design, and subsequent analysis used, restricts the significance of absolute ED_{50} values to three drug-common control areas. Loosening of this restriction could be accomplished by the rather more laborious simultaneous estimation of three parameters,[20] including the limiting spirochetemia sampled by the controls.

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the next two hours. Hence, maximum suppressive activity becomes manifest considerably later than peak blood concentrations of either drug would be expected to occur in infected animals. Whether this delay reflects the time required for the drugs to act or for phagocytosis and disintegration of spirochetes to occur is unknown. Stained blood smears scarcely permit differentiation between viable and damaged or killed organisms. Immobilization of *B. novyi* suspended in 50% horse or beef serum occurred with chloramphenicol at concentrations of 10 to 50 μg/cc within two hours at 37 C. This effect was not observed regularly with penicillin G at concentrations extending up to 600 μg/cc (unpublished data by the authors). These observations indicated that chloramphenicol either is more active or acts more rapidly. Such difference between drugs in amount of or in time required for action are not now apparent in vivo. Suggestions of more rapid action by penicillin G provided by the data in figure 2 must be considered in relationship to the relative suppressive activities of the drugs on a weight basis.

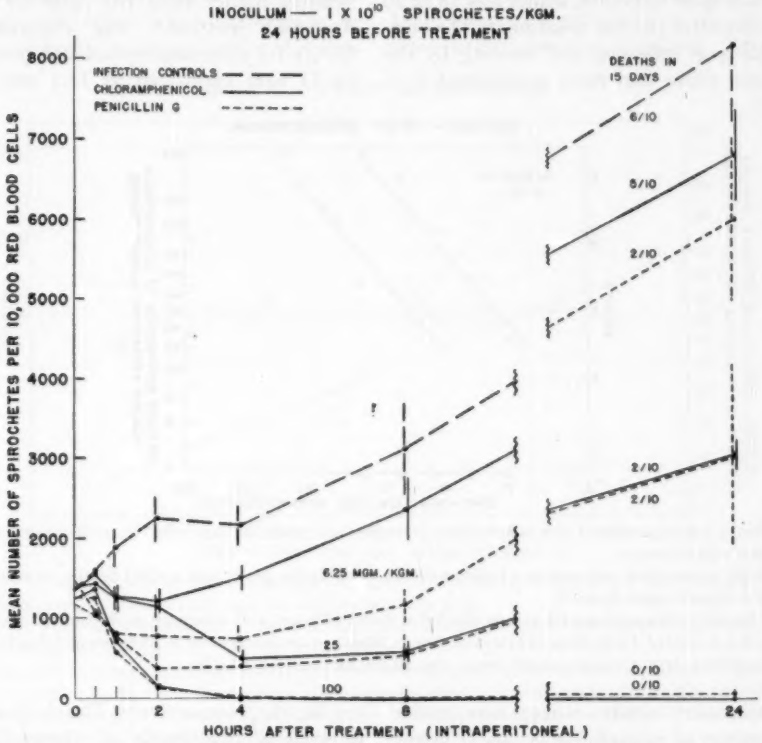

FIG. 2.—Effects of single intraperitoneal doses of chloramphenicol and of penicillin G on the immediate course of the spirochetemia.

Standard errors are indicated by vertical lines drawn through the loci of the means, with the magnitude of a standard error represented by the segment either above or below a locus.

Comparison of actions on a schedule of multiple injection of drug.—Groups of 10 mice were infected as in preceding experiments with 1×10^{10} spirochetes/kg and were treated intraperitoneally at 4, 22, and 28 hours after the inoculation of spirochetes. Smears were made 46 to 48 hours after infection, which was 18 to 20 hours after the last treatment. The suitability of infecting and treating by the same route had been established in a preliminary study, where comparable numbers of spirochetes occurred in the circulating blood three hours after inoculation with 1×10^{10} spirochetes/kg whether inoculation was by the intravenous or by the intraperitoneal route.

The results are shown in figure 3 and legend, where, as in the case of the diet experiment, there was a substantial linear component of the log dose-probit suppression relationship for each drug. Suitable computations indicated the slopes and variances of these regressions not to be significantly different. The average slope for the two drugs, shown in the figure, and an over-all potency ratio thus again express the essential features of suppressive action. The potency of chloramphenicol relative to penicillin G by this parenteral comparison was 0.37 (95% confidence limits, 0.28–0.49), which is perhaps significantly higher than the ratio by the drug-diet method.* The suppressive ED_{50}'s for chloramphenicol and penicillin G were estimated at 15.1 and 5.7 mg/kg/day, respectively. The similarity in general magnitude of these ED_{50} values and those by the diet method are

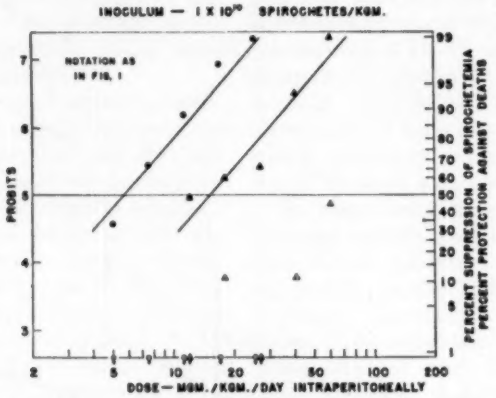
INOCULUM — 1 X 10¹⁰ SPIROCHETES/KGM.
NOTATION AS IN FIG. 1
PROBITS
PERCENT SUPPRESSION OF SPIROCHETEMIA
PERCENT PROTECTION AGAINST DEATHS
DOSE — MGM./KGM./DAY INTRAPERITONEALLY

FIG. 3.—Comparison of chloramphenicol (triangles) and penicillin G (circles) by multiple intraperitoneal administration.

Solid marks show suppression of spirochetemia, open marks protection against deaths, as noted in and computed as in figure 1.

The daily intraperitoneal dose was divided at 4 and 22 hours with reference to the time of inoculation for a total of 3 injections (1½ days or doses). Smears were taken at 46 to 48 hours and deaths observed for 7 days. Control spirochetemia was 10,820 and survivals 1/10.

* The untreated mice and those treated with penicillin G ranged from 18 to 20 g, while those treated with chloramphenicol ranged from 15 to 17 g. Thus, there exists some possibility of bias in the ratio and invalidity in the computed confidence range. However, rather extensive experience has failed to provide evidence of any influence of animal size on the level of blood infection, not corrected by the usual adjustment of inoculation according to body weight. The remote possibility of some influence related to other than the first power of host size on effective drug concentration remains.

interesting in view of the differences in treatment regimes.

No evident protection against deaths occurred with penicillin G even though all doses caused significant suppression of spirochetemia. Although protection occurred with the higher doses of chlorampenicol, doses of either drug adequate for appreciable suppression of spirochetemia did not confer a corresponding degree of protection. By comparison with the drug-diet experiments, severe discontinuity or briefness in regime apparently handicaps vital protection more than it does blood-parasite suppression.

Effects of treating more uniformly lethal infections by more prolonged multiple injection of drugs.—The primary purpose of this phase of the study was to provide a more informative comparison of drugs in protecting against death. Two experiments were done, involving a total of 250 mice. More severe infections than in the preceding experiments were induced by the injection of 5×10^{10} spirochetes/kg. The first injections of drug were given at 4 and 22 hours after infection. Each daily subsequent dose was similarly divided at the 4th and 22nd hour with reference to the time when infections were initiated. Treatment was continued for 9½ days, thus including a total of 19 injections. Smears were taken at 46 hours, just preceding the fourth drug injection. The main differences between this experiment and the preceding were the higher infection level and longer period of treatment. Mortality data were collected during 30 days after infection. In each experiment all untreated mice died within 7 days.

Certain observations provided reasonable assurance that mortality among

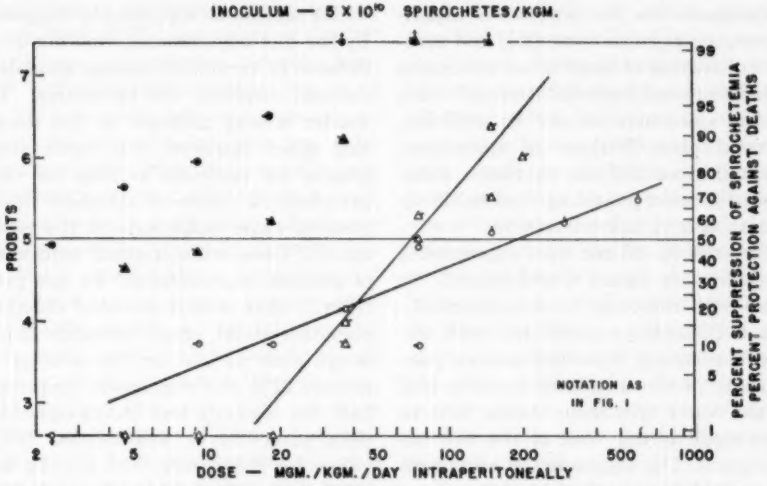
INOCULUM — 5 X 10¹⁰ SPIROCHETES/KGM.
PROBITS
PERCENT SUPPRESSION OF SPIROCHETEMIA
PERCENT PROTECTION AGAINST DEATHS
NOTATION AS IN FIG. 1
DOSE — MGM./KGM./DAY INTRAPERITONEALLY

FIG. 4.—Comparison of chloramphenicol (triangles) and penicillin G (circles) by sustained multiple intraperitoneal administration, against a higher infection level.

Solid marks show suppression of spirochetemia, open marks protection against deaths, as noted in and computed as in figure 1.

The daily intraperitoneal dose was divided into two treatments per day, similar to those of figure 3, but extending for 9½ days. Smears were taken 46 hours after infection, and deaths were observed for 30 days. Control spirochetemia was estimated at 14,768 for the one experiment in which it was studied, and control survivals were 0/10 and 0/13 for the two experiments.

treated animals was not importantly complicated by drug toxicity or by concomitant infections. In the second experiment no deaths occurred among groups of uninfected mice given chloramphenicol or penicillin G on the same schedule and at the maximum levels used with infected mice, namely, at 200 and 600 mg/kg/day, respectively. This amount of chloramphenicol is, however, near the maximum tolerated dose.[25] Subsequent to the protection experiments, appropriate anaerobic and aerobic cultivation of heart blood specimens failed to reveal bacterial contamination of the spirochete strain. In addition, sintered glass filtrates of spirochete-infected blood did not contain a lethal agent for mice, providing reasonable exclusion of a virus contaminant.

The results of the two experiments are shown in figure 4 and legend. At these high infection levels, spirochetemia is difficult to quantitate with desirable accuracy from the smears, particularly in the untreated controls and at the lower treatment doses. Smears were made in only one of the two experiments. The suggestion of curvature in trend of suppression with dosage seen in the figure may well have resulted from a poor estimate of the limiting (control) spirochetemia. It was, therefore, not considered advisable to attempt precise analysis of the suppression data. Nevertheless, inspection of the plotted points yield, again, apparent parallelism in behavior and 50% suppressive dose estimates roughly comparable with those from the preceding experiments with less dense parasite populations.

As in the preceding parenteral experiment, much higher doses were required to protect against deaths than to suppress the blood parasites, and again this discrepancy is greater for penicillin G. Both drugs did exhibit important protection against deaths when relatively large doses were used. Analysis of these data by probit methods[20,23] showed reasonable homogeneity for data combined from the two experiments for each drug.

It is important to note that the slopes of the two lines were different ($P = 0.005$), which means, as was already suggested by the diet experiments, that the drugs differed in variability among animals in amount required for protection. The scatter among animals, in the dosage (log dose) required, was three times greater for penicillin G than for chloramphenicol (ratio of standard deviations or slope reciprocals on the probit chart). Thus, while a small percentage of animals was protected by less penicillin G than was required of chloramphenicol, about equal amounts of the drugs were needed on the average to protect 22% of the animals. To protect half the animals less chloramphenicol than penicillin G was needed (PD_{50} values for the former, 70.1 mg./kg/day [95% limits, 55.4 to 89.1] and for the latter, 189 mg/kg/day [109 to 439]). To insure protection of a high percentage of such infected mice, much less chloramphenicol than penicillin G would be needed.

Attempts to determine whether the mice treated with large doses of drug and surviving for 30 days were cured did not yield definitive results. Thirty-one mice protected with chloramphenicol and 15 protected with penicillin G were given a challenge dose of 1×10^{10} spirochetes/kg 36 days after initial infection. All were refractory to reinoculation, but there is uncertainty whether refractoriness to reinoculation is indicative of residual infection or is due to immunity persisting after cure of the

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initial infection.[8] Subinoculations of tissues or blood were not done, since in preliminary work on untreated mice, such procedures approximately one month after infection did not consistently reveal latent infections.

Observations on survival time of unprotected animals.—Throughout the entire investigation with inoculums of 1×10^{10} spirochetes/kg, there was no reliable evidence of sensitivity of survival time of unprotected mice to drug or to dose. With inoculums of 5×10^{10} there was a progressive increase, from low to high dosage of either drug, of 44 to 93% in mean survival time of unprotected animals; but this was principally due to delay in unusually early deaths occurring at this infection level. The upper limit of the time of death was not apparently related to therapy, that is, there was a limiting period beyond which the typical animal was safe from the acute phase of the infection. If treatment failed or infection level was high, it might die earlier but not later. The mode of this upper limit for those dying out of groups of 10 or 13 animals was 5 to 6 days, whether the observation period was 7 or 15 days with inoculums of 1×10^{10} or 30 days with inoculums of 5×10^{10}. In the latter case, where the protection percentages were analyzed quantitatively, it is gratifying that no deaths occurred after the 9th day of a 30-day observation period and that treatment was carried completely through the period of possible usefulness.

Fairly regular development of acquired immunity is indicated by the characteristic limiting time of death.

DISCUSSION

As a convenient point of departure for exploring the action of chloramphenicol against relapsing fever and comparing it with that of penicillin G, arbitrary procedures which probably were not optimal for determining the full potentialities of either drug have been employed. Hence, quantitative interpretation of the results are restricted by the scope of the study.

Comparison of the drugs on the basis of the suppression of an acute spirochetemia suggests qualitative similarity in action, while comparison in terms of protection against death indicates dissimilarities. Although more chloramphenicol than penicillin G was required for a given degree of suppression, similarity in manifest quality of action was observed when infections of different intensities were treated parenterally with single or multiple injections of drugs and when a single infection level was treated by the diet method. Available data on the course of blood levels of chloramphenicol and of penicillin G following parenteral administration in normal mice help to account for similarity in such aspects of drug action as the time required for detectable suppressive effect, the duration of effect, and the slopes of the dosage-suppressive effect curves.

Not only were the dosage suppression curves substantially parallel for the two drugs at a given infection level or treatment schedule, but, by inspection, the pairs of curves are seen not to differ significantly in general trend among the infection levels and treatment schedules. Further, remarkably similar 50% suppressive doses resulted with inoculums differing fivefold and with corresponding 48-hour control spirochetemias differing about twofold. This leads to a fundamental consideration which seems worth mentioning: The suppression of a multiplying population of blood parasites may be considered a quantal phenomenon, with individual parasites possessing varying tolerances for the drug. Tolerances in general will be in-

fluenced in an unknown direction by fluctuations in concentration and the time factor of drug action; and variability in tolerances will be affected by such factors as intra- and interhost differences in defenses and drug metabolism. A dosage suppression trend such as those of figures 1, 3, and 4, on a probit scale, may be regarded as a rectified cumulative distribution curve of tolerances for such a pooled, growing population of parasites. The reciprocal of the slope of the probit regression is the standard deviation of tolerances. It is thus not surprising to find similar median tolerances and slopes in experiments with varying infection levels (fig. 3 vs. fig. 4), because these statistics of a sample do not depend on sample size (number of parasites). Finally, the implication is that the spirochetes dealt with here have different mean but similarly variable tolerances (log dose) to penicillin G and to chloramphenicol.

In contrast with the qualitative similarity of action of the two drugs on free blood spirochetes is their dissimilarity in protecting animals against death. Unknown variable factors operate between suppression of blood parasites and protection against vital aspects of the general infection. Comparison of variability in blood parasite tolerances and variability in vital requirements of drug by individual hosts is thus not instructive. However, two drugs which are so similar in suppression and so dissimilar in protection must possess differences in uniformity of effective access, temporal or spatial, to the vital aspects of the infection. Apparently by administration in the diet at the low infection level, and more certainly by grossly discontinuous parenteral administration at the high infection level, vital protection by chloramphenicol was less variable than was that by penicillin G.

The present observations on the protective action of penicillin G are not necessarily contradictory to those of Richardson et al[8] since in the present work a more uniformly lethal infection was established and the treatment schedule was different. Actually, comparable parts of the two studies produced results which agreed reasonably well.

Several investigations on experimental relapsing fever[26–29] have described the inefficiency of penicillin in curing brain involvement except when administered intracranially. This, of course, may be explained by the finding of many workers[30] that penicillin frequently does not readily diffuse from the blood into the cerebrospinal fluid. Studies on the tissue distribution of chloramphenicol[31] demonstrated high concentrations in the liver, kidneys, lungs, and spleen, with appreciable but somewhat lower levels in the brain of various laboratory animals and in the cerebrospinal fluid of man. Possibly the less variable protection of mice by chloramphenicol in the present work may have resulted from superior diffusion into, or less interference in, those tissues vitally affected by *B. novyi*. However, it must be pointed out that the radical curative actions of chloramphenicol and of pure penicillin G have not been evaluated.

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Clinical trial should not be discouraged by the dosage of either drug necessary for therapeutic effect in experimental studies, which for convenience are conducted with massive infections. From laboratory studies it was predicted that impractical doses of penicillin would be required for treating human relapsing fever.[32,33] Actually this drug has proved to be clinically effective against both louse- and tick-borne relapsing fever in man[34–37] and in doses relatively much smaller than were required for demonstrating its therapeutic effect in rodents.[32,38,39]

SUMMARY AND CONCLUSIONS

Chloramphenicol was effective against infections of *Borrelia novyi* in mice. Treatment of standardized acute infections by the diet method or by intraperitoneal injection resulted in suppression of the blood infection and reduction in mortality among infected animals.

With suppression of the blood infection as a criterion of activity, chloramphenicol and penicillin G exhibited similar dose-response relationships, but larger doses of chloramphenicol were required to produce a given effect. The potency ratio of chloramphenicol to penicillin G with treatment by the diet method was 0.2 and by intraperitoneal injection was (twice per day) 0.37.

Differences in quality of action arose from comparison of the drugs in protecting mice against the lethal effects of the infections. The variability among animals in dosage (log dose) required for protection was significantly and considerably less with chloramphenicol. Thus, while greater, but less variable amounts of chloramphenicol were required to protect animals against approximately 50% lethal infections or to protect a small percentage of animals against more uniformly lethal infections, less chloramphenicol than of penicillin G was required to protect 50% or more of the animals against more uniformly lethal infections.

Data on plasma levels of chloramphenicol and of total nitro compounds in normal mice reveal rapid absorption and rapid degradation or excretion following parenteral or oral administration of the drug.

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VISCOSIMETRIC MEASUREMENT OF STREPTOCOCCAL ANTI-HYALURONIDASE: COMPARISON WITH TITERS OBTAINED BY TURBIDIMETRY

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It has recently been observed that the mean titer of neutralizing antibodies to streptococcal hyaluronidase is greater in the serum of patients with active rheumatic fever than of patients convalescing from acute streptococcal infections, and that the range of such titers in the former group extends above the range found in the latter.[1–3] Of the three physicochemical reactions by which one can measure the effect of hyaluronidase on hyaluronic acid—the mucin-clot reaction,[4–6] turbidimetry,[7,8] and viscosimetry[9]—only the first named was used in the work referred to above. For two reasons it seemed indicated to study this phenomenon by the other two reactions. First, the mechanism of the mucin-clot reaction of hyaluronic acid is not understood, and, second, it is at present an open question whether the depolymerizing agents measured by the three physicochemical reactions are in reality identical or distinct enzymes. Although the great majority of comparative studies of various effects of hyaluronidase have involved the comparison of one physicochemical method with a biological or chemical method of assay (such as spreading potency in skin or liberation of reducing sugars from the substrate), some work has been done on comparison of physicochemical meth-

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* Fellow of the National Institute of Health.

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ods. McClean[10] found a parallelism between titrations of various preparations of hyaluronidase by mucin-clot-prevention (M.C.P.) and reduction of viscosity. The ratio of titers as obtained by these methods varied rather widely, but a good coefficient of correlation was obtained. Pike[11] found some correlation between M.C.P. and turbidity-reduction measurements of testicular hyaluronidase. However, Meyer[8,12] reported preparations of hyaluronic acid in which some steps of purification had apparently destroyed some bonds of polymerization necessary for the mucin-clot reaction without affecting the reaction of turbidity, and postulated that different sites of action were involved in the digestion of hyaluronic acid.

Accordingly, it was decided to study the concentration of streptococcal antihyaluronidase in the serum of patients with active rheumatic fever and relevant control groups by turbidimetry and viscosimetry, in the interest either of confirming the earlier work which had been done by mucin-clot-prevention or of detecting and measuring the antibody to another streptococcal enzyme if different hyaluronidases were indeed involved in the respective reactions. The development of a technique for measurement of serum antihyaluronidase by turbidimetry has been reported elsewhere,[13] as well as the confirmation of the differences between rheumatic and poststreptococcal serums which had been found by M.C.P.[14] The present paper describes a method for measuring neutralizing antibodies to streptococcal hyaluronidase by the reduction of viscosity of the substrate and the application of this method to the serologic problem mentioned above.

MATERIALS AND METHODS

Hyaluronic acid.—The substrate used in these measurements of hyaluronidase and of neutralizing antibodies thereto was prepared by a method described elsewhere.[14] In essence this method involves the preparation of crude hyaluronic acid by a slight adaptation of the method of McClean[15] followed by a step of purification which involves aqueous extraction of the crude substrate and reprecipitation in potassium acetate-alcohol. This preparation of hyaluronic acid was shown to be quite pure by tests for the concentration of nitrogen (2.7 to 2.8%), phosphorus (less than 0.04%) and other tests.

Hyaluronidase.—The streptococcal hyaluronidase used in this work was prepared as described in an earlier study.[16] It was produced by cultivating a strain of hemolytic streptococcus in a dialysate medium relatively free of protein and concentrating the culture supernate. The final form of the preparation of enzyme was a dry powder, which retained its potency during many months of storage at 4 C.

The testicular hyaluronidase used for comparative studies was prepared from bovine testis by the method of Madinaveitia.[17] This material was

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generously supplied by the Wyeth Institute of Applied Biochemistry.

Clinical material.—The children whose serums were examined in this study included presumably normal children; convalescents from hemolytic streptococcal infection, in almost every case scarlet fever; and patients with active and inactive rheumatic fever. The last named were patients in the institutions from which this work is reported. The diagnosis of active rheumatic fever was made on the basis of repeated clinical and laboratory examination, and only children with definite evidence of rheumatic carditis were classified as having active rheumatic fever. Further clinical details are given elsewhere.[5]

The measurement of relative viscosity.—The measurement of the degree of neutralization of hyaluronidase by antibodies depends on a method of measuring the enzyme, and the latter, in turn, on a method of measuring the property of the substrate. For the present study, the number of determinations of relative viscosity of solutions of hyaluronic acid was seen to be so great as to preclude the use of the classical Ostwald viscosimeter. Accordingly, a method was devised which depends on the incubation of enzyme and substrate, or of serum, enzyme and substrate in serologic tubes, then the transferring of the mixture into a serologic pipette to which a length of fine glass capillary is affixed, and, finally, the observation of the time required for a given volume of the solution to flow out of the pipette through the obstruction offered by the glass capillary. The details are as follows:

For the measurement of the relative viscosity of a solution of hyaluronic acid, 0.6 cc of this material dissolved in buffered saline solution was added to an equal volume of the diluent alone. This mixture was drawn into a 1 cc serologic pipette to a point well above the 0.6 cc mark. A bit of rubber tubing was used to join the lower end of the pipette to a 7.75 cm length of glass tubing, with a bore of 0.5 mm. The pipette was now placed in a vertical position and held thus by two clips fixed to a vertical bar of wood. By means of a stop watch the precise interval of time was determined during which the meniscus of the hyaluronic acid solution passed from the 0.6 cc to the 0.8 cc marks. The constancy of this interval was found to be quite satisfactory on repetition of given determinations in the same or different pipettes. The solution in the pipette was allowed to drain into the same tube from which it was drawn, so that any number of repetitions of a given measurement of relative viscosity was possible in the case of each specimen, although usually only one was necessary. The lengths of glass capillary were washed between determinations by connecting one end to a suction hose and dipping the other end rapidly several times in diluent. It was not found necessary to dry the capillaries by drawing alcohol and ether through them after the water. In a given experiment calibration curves were constructed by measuring the time of flow of several known concentrations of hyaluronic acid and plotting these times of flow aginst the respective concentrations. From such curves the unknown concentrations of hyaluronic acid could be determined by interpolation, after determining the time of flow of these solutions. The diluent mentioned above consisted of an 0.85% solution of sodium chloride, buffered at pH 7.4 with phosphate at a final concentration of 0.02*M*. This saline solution was used as a diluent for all reagents.

For the measurement of the activity of hyaluronidase, 0.6 cc of a 0.4% solution of hyaluronic acid was incubated with 0.2 cc of a dilution of the enzyme to be tested and 0.4 cc of saline solution. After 20 minutes of incubation at 37 C, the tubes were transferred to another water bath, at 60 C, in order to inactivate the enzyme. After 10 minutes at 60 C and an interval to allow a return to room temperature, the relative viscosity of the mixture was measured.

For measurement of the neutralizing titer of a given serum, 0.4 cc of serial twofold dilutions of the serum in saline solution was incubated for 15 minutes at room temperature with 0.2 cc of a solution of streptococcal hyaluronidase of a given concentration. Thereafter the effect of the residual, unneutralized hyaluronidase was measured as described above.

RESULTS

1. The viscosity of the substrate.—The viscosity of solutions of the preparation of hyaluronic acid used was measured at several concentrations both by the classical Ostwald viscosimeter and by the method described above. Figure 1 shows graphically the relationship between the concentration of solutions of hyaluronic acid and the values of relative viscosity corresponding to these concentrations. Figure 2 shows the correlation between measurements as made by the two methods at various concentrations of the substrate.

In figure 1 it is seen that the relative viscosity of solutions of hyaluronic acid increases in a uniform manner with the

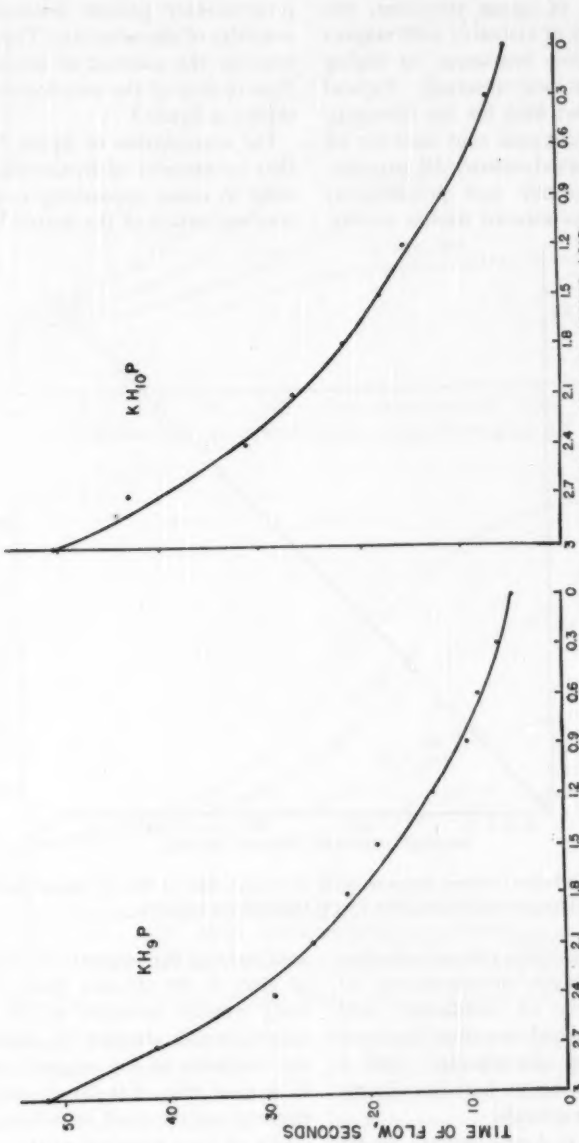

FIG. 1.—The time of flow of solutions of hyaluronic acid at various concentrations (two preparations).

concentration of those solutions, the rate of increase of viscosity with respect to concentration increasing as higher concentrations are reached. Typical curves are shown here for the two preparations of hyaluronic acid used for all the work reported below. All preparations of hyaluronic acid prepared as described above showed similar curves.

progressively greater decreases in the viscosity of the substrate. Typical curves relating the amount of enzyme to the time of flow of the reaction mixture are shown in figure 3.

On examination of figure 3 it is seen that an amount of hyaluronidase sufficient to cause apparently complete depolymerization of the native hyaluronic

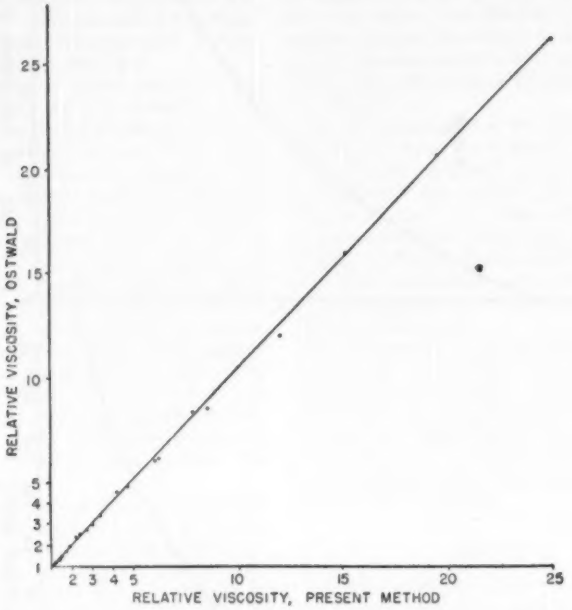

FIG. 2.—The correlation between measurements of relative time of flow by the method described here and determinations of relative viscosity by the Ostwald viscosimeter.

Figure 2 shows that a linear relationship exists between measurements of relative viscosity of hyaluronic acid made by the method described here and by the Ostwald viscosimeter, with a rather close correlation between the results of the two methods.

2. The effect of hyaluronidase on the viscosity of the substrate.—Incubation of constant amounts of hyaluronic acid with hyaluronidase showed that increasing amounts of the enzyme caused

acid reduces the viscosity of the mixture to that of the diluent itself. Progressively smaller amounts of the enzyme cause smaller degrees of change from the viscosity of the original substrate. It is seen also that larger amounts of enzyme are required to reduce the viscosity of large amounts of the substrate to a given low value of viscosity.

3. Quantitative relations between enzyme and substrate.—In a study of the hyaluronidase-hyaluronic acid system

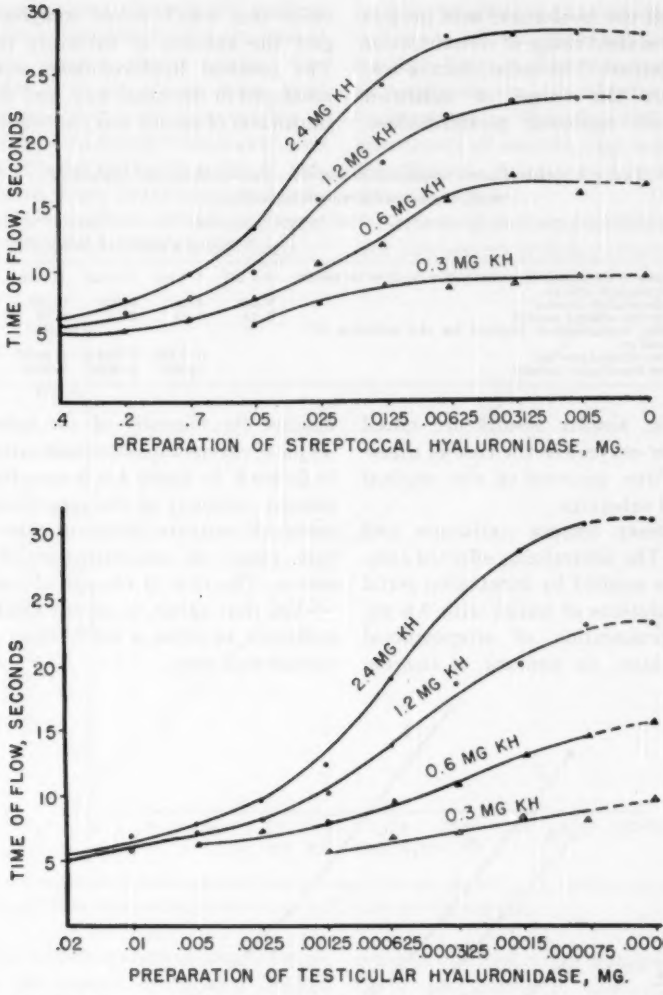

FIG. 3.—The effect of hyaluronidase on the relative viscosity of solutions of hyaluronic acid.

by turbidimetry[13] it was shown that the quantitative relations between the two reagents were consistent with those of a monomolecular enzyme reaction. In order to determine whether such relationships could be shown by viscosimetry, data were collected from 7 experiments such as the ones presented in figure 3 (6 experiments with streptococcal hyaluronidase and one with testicular enzyme), and the amounts of enzyme were determined which were necessary for reduction of the substrate to a given fraction of the original amount. The mean values are shown in table 1.

On examination of table 1 it is seen that the same amount of streptococcal hyaluronidase was required to depoly-

merize half the hyaluronic acid present through a sixfold range of concentration of the substrate. The same effect is seen throughout the range of substrate tested with testicular hyaluronidase, twice that which would completely digest the amount of substrate present. The residual hyaluronidase was then measured in the usual way, and the concentration of serum was plotted directly

TABLE 1.—*Quantitative relationships between hyaluronidase and hyaluronic acid, as measured viscosimetrically.*

	Original amount of hyaluronic acid				
Mg of preparation of streptococcal hyaluronidase required for the reduction of hyaluronic acid to:	3.6 mg	2.4 mg	1.8 mg	1.2 mg	0.6 mg
one-half the original amount	0.043	0.041	0.039	0.040	0.038
one-quarter the original amount	0.13	0.13	0.13	0.10	
Mg of testicular hyaluronidase required for the reduction of hyaluronic acid to:					
one-half the original amount		0.00095	0.00088	0.00095	0.00091
one-quarter the original amount		0.0038	0.0033	0.0035	

and, again, similar results are noted with either enzyme in the case of digestion of three quarters of the original amount of substrate.

4. Relations between antiserum and enzyme.—The neutralizing effect of antiserum was studied by incubating serial twofold dilutions of serum with 0.6 mg of our preparation of streptococcal hyaluronidase, an amount of enzyme against the viscosity of the substrate. Typical curves thus obtained are shown in figure 4. In figure 4 it is seen that the relative viscosity of the serum-enzyme-substrate mixture decreases over a certain range of concentration of each serum. The rate of change of viscosity within that range of serum dilution is sufficient to allow a fairly clear determination of titer.

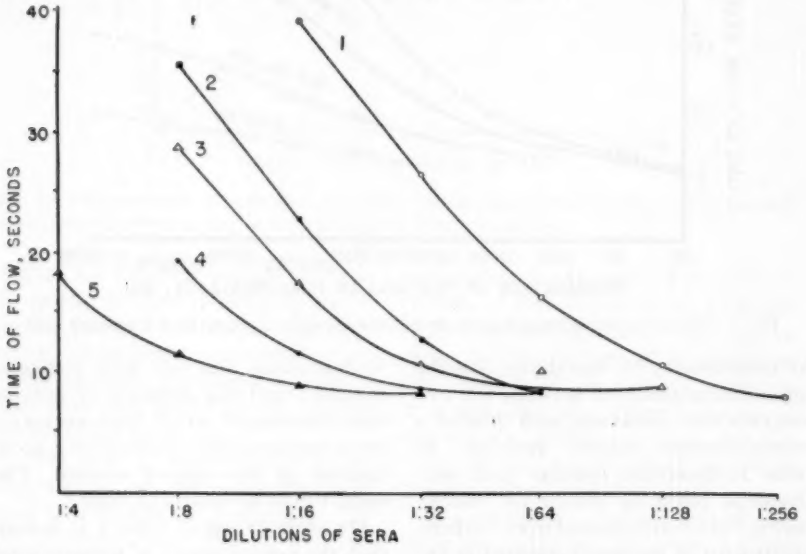

FIG. 4.—The neutralization of streptococcal hyaluronidase by antibody, as measured by viscosimetry.

On repeated examination of a number of serums it was found that the curves of serum dilution versus relative viscosity for a given serum were most closely grouped at a viscosity reading of 15 seconds. Accordingly this was chosen as the end point of the titration. At this end point 0.56 mg of the original 0.6 mg of the preparation of streptococcal hyaluronidase have been neutralized.

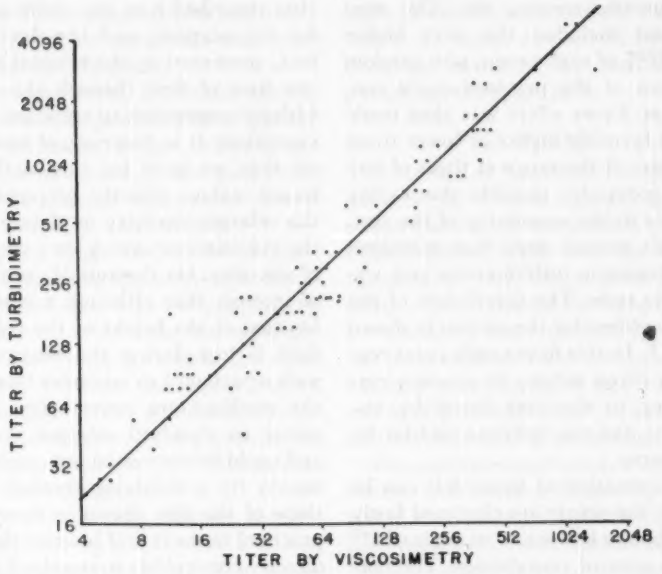

FIG. 5.—Streptococcal antihyaluronidase in human serums derived from various clinical groups: correlation of titers observed by turbidimetry with those by viscosimetry.

5. Viscosimetric antihyaluronidase titers in the serums of various clinical groups.—The cost in time and materials of performing antihyaluronidase tests by viscosimetry on serial dilutions of great numbers of serums was prohibitive. However, considerable numbers of serums of the clinical groups of interest to this investigation were available, with streptococcal antihyaluronidase titrations done by the mucin-clot-prevention and turbidimetric tests. In order to determine whether the same relative distributions of streptococcal antihyaluronidase titers in rheumatic and streptococcal disease could be observed by viscosimetry as by the other two tests, an assortment of serums were selected for simultaneous testing by turbidimetry and viscosimetry.

Because of the comparative nature of this experiment it was particularly important that all titers obtained be entirely comparable to each other. The following precautions were therefore taken. First, a given pool of γ globulin derived from human serum was used as a standard serum, appearing in every rack of tubes of each day's test. Second, serums of each clinical category, normal, rheumatic and poststreptococcal, were repeated in each day's test from that of the day before. Third, each clinical category was represented through-

out the range of antihyaluronidase titers existing in that group. Finally, each day's test was composed of serums from each of the clinical groups in the same relative numbers as the size of the entire group and in the same relative range of titers. Thus, the tests on the first of the 5 days required for this experiment included the lowest titered 20% of the normal serums to be examined, the lowest titered 20% of the active rheumatic serums, etc. The next day's test included the next higher titered 20% of each group, plus random repetitions of the previous day's run, and so on. Every effort was thus made to avoid favoring higher or lower titers in any part of the range of titers of any clinical group by possible day-to-day variations in the sensitivity of the test. Ninety-six serums were thus examined by simultaneous turbidimetric and viscosimetric tests. The correlation of the respective titers for the serums is shown in figure 5. In this figure each point represents a given serum, its abscissa corresponding to the titer found by viscosimetry, and the ordinate its titer by turbidimetry.

On examination of figure 5 it can be seen that the points are clustered fairly closely about a line set at an angle of 45° with the axes of coordinates. The correlation is apparently quite high, with proportionate increases from serum to serum between titers as determined by the two methods.

DISCUSSION

1. The measurement of relative viscosity.—It will be obvious to anyone familiar with the Ostwald viscosimeter that the numbers of determinations of relative viscosities involved in these experiments would have been altogether impossible with that instrument. The method described here was devised in an effort to obtain some measurement or reading for each solution which would, at least, increase with increasing viscosities of such solutions. With the aid of calibration curves prepared for known concentrations of hyaluronic acid such readings of solutions of unknown concentrations could then be converted into concentrations of residual, undigested hyaluronic acid. The measurement of the time of fall of 0.2 cc of solution through a simple device such as that described here was quite adequate for the purpose, and the data are, in fact, presented in the original form, as the time of flow through this system, without conversion to standard relative viscosities. It is, however, of some interest that we have by this method obtained values directly proportional to the relative viscosity as determined in the viscosimeter over a very wide range of viscosity. On theoretical grounds it is of interest that although a substantial fraction of the height of the column of fluid is lost during the measurement, with no attempt to minimize this change the readings are nevertheless proportional to standard relative viscosities and could be converted into such values merely by multiplying through by the slope of the line shown in figure 1. In practical terms it is of interest that data directly convertible to standard relative viscosity can be obtained with a smaller specimen and much less costly equipment and in a small fraction of the time involved in determinations by the Ostwald viscosimeter. As a matter of fact, it is entirely probable that even the need for a factor for converting our times of flow to values obtained by standard instruments could be obviated simply by choosing different end points than the 0.6 and 0.8 graduations of the pipette, since at another arbitrarily chosen part of the pipette—0 to 0.2 cc—readings were obtained which were again directly proportional to those obtained

with the same solutions in the Ostwald viscosimeter. The factor of proportionality was, of course, different, as might be expected in view of the different head of hydrostatic pressure. Selection of the proper interval on the 1 cc pipette could, then, make this a direct reading instrument for standard relative viscosities as obtained with the Ostwald apparatus. (It may be relevant to note that the Ostwald viscosimeters themselves, as commercially available, show slight differences which are detectable with relatively viscous solutions.)

2. The viscosimetric antihyaluronidase titers in serums of patients with streptococcal and rheumatic disease.—The numbers of serums in each clinical group and among the normal subjects were not such as would be used to establish the comparative range and mean of antibody titers among those groups. However, it is felt that with the precautions noted above in the selection arrangement of serums to be tested the close correlation between the sets of titers shown in figure 5 offer a direct confirmation by viscosimetry of the results we have obtained by the M.C.P. reaction and by turbidimetry.

3. The relationship between the enzyme measured by viscosimetry and that measured by turbidimetry.—The correlation between antihyaluronidase titers as derived by these two methods of measuring hyaluronidase as shown in figure 5 offer some indirect evidence on the question of the identity or distinction of the enzymes so measured. If different hyaluronidases were involved it would be quite unlikely that such enzymes had been liberated by the hemolytic streptococcus in such nearly proportional amounts into the tissues of the 96 subjects during their recent contact with that organism, and that these children had produced neutralizing antibodies to two or more hyaluronidases in such proportionate amounts as to have produced an essentially constant relationship between the concentrations of the antibodies measured in each serum. Accordingly, these data would be consistent with a unitarian concept of hyaluronidase, as measured by the available physicochemical methods.

SUMMARY

A method is described for the determination of relative viscosity which requires less time and materials than such measurements made with the Ostwald viscosimeter and which gives readings directly proportional to those obtained by the latter instrument. This method has been applied to the measurement of hyaluronic acid, and, in turn, the measurement of streptococcal hyaluronidase and of neutralizing antibodies thereto.

Representative serums from groups of normal children and those with streptococcal and rheumatic infection showed a high degree of correlation between antihyaluronidase titers as determined by viscosimetry and by turbidimetry. These data offer a confirmation of the comparative titers of these antibodies which had been found by other methods and some evidence against a multiplicity of streptococcal hyaluronidases as measured by physicochemical means.

ATTEMPTS TO CURE PARAKEET PSITTACOSIS CARRIERS WITH AUREOMYCIN AND PENICILLIN

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Prevention of human psittacosis ultimately depends on eliminating carriers among the diverse avian species so highly treasured by bird lovers. Apparently healthy but virus-spreading birds maintain infection in breeding establishments and only too often bring the disease into the human household. Although experience has shown that the spontaneous carrier rate in commercial parakeet flocks is quite variable and frequently low —2 to 5% (Meyer and Eddie, 1947), the damage inflicted is by no means negligible. The livelihood of many people could be assured and protected provided they could offer for sale psittacine birds, pigeons and fowl free from the psittacosis agent.

Ever since penicillin proved effective in the treatment of experimental and human clinical psittacosis (Meyer, 1949), efforts have been made to effect a cure in chronic infections. Invariably, irrespective of dose, intracellular parasites were not affected by antibiotic (Smadel and Jackson, 1948) or other drugs (Hurst, 1948; Meiklejohn et al, 1946; Early and Morgan, 1946). Virus could readily be isolated from the viscera by subinoculation with emulsified liver and spleen or kidney and intestinal content into mice.

Recently, Wong and Cox (1948) reported that small doses of aureomycin completely protected mice infected intraperitoneally with yolk-sac suspensions of 47,000 LD_{50} of the psittacosis viral agent, and in infected embryonated hens' eggs this antibiotic not only prevented death of the embryos but inhibited formation of elementary bodies. In pneumonic infections, Loosli (1949) found that mouse lungs were completely clear of the virus of mouse pneumonitis when treatment with this drug was given for 7 days.

It was reasoned that through a scheduled course of intensive treatment with aureomycin it might be possible to eradicate the organism from the tissues of parakeet psittacosis carriers. It is known that as a sequel to experimental infection with highly active virus, approximately 50% of a susceptible strain of parakeets may become carriers one or two months after inoculation. Such artificially produced carriers are suitable for chemotherapeutic studies. Herein are presented data concerning an unsuccessful attempt to accomplish elimination of the viral agent by treating a series of birds with aureomycin and another series with penicillin.

Fifty-three mature and immature parakeets were infected by intramuscular injection of 0.5 ml of a 10^{-4} dilution of psittacosis-infected yolk-sac material (strain 6BC). One died on the 9th day with typical lesions. After 37 days, when the birds did not appear sick, 5 were sacrificed and only 3 were found to be infected (virus recovered from all 3; 2 had gross lesions). In an effort to be certain that more birds became sick and subsequently carriers, a second injection consisting of 0.5 ml of a 10^{-3} dilution of yolk-sac tissue infected with the Seattle strain of parakeet origin was given to the remaining 47 birds on the 51st day after the first inoculation. Three of the birds succumbed after this second injection.

All of the remaining birds appeared healthy 19 days after the second inoculation. At that time, 5 were sacrificed to determine the carrier status. The viral agent was isolated from all 5. Therefore, 2 groups of 10 each were then selected at random from the remaining 37 and segregated for treat-

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ment with antibiotics. Penicillin in aqueous physiologic saline solution was then given intramuscularly to one group of 10 birds in a dose of 1000 units per bird twice a day for 10 days. Aureomycin solution, freshly prepared at each dosing time, was similarly injected in 0.4 mg doses into the other 10. The parakeets, which weighed about 30 g each, were all clinically well throughout the therapeutic period. There were no local reactions from these drugs.

On the 25th and the 29th day after treatment was stopped, all the parakeets were sacrificed and carefully autopsied. The spleens were measured and 10% emulsions of organs were used to test for the presence of the viral agent by the usual mouse passage method. Results are given in table 1.

TABLE 1.—*Results of treatment of psittacosis carriers (parakeets) with penicillin and aureomycin.*

Penicillin				Aureomycin				Controls			
Parakeets	Size of spleen, mm	Isolation of virus*		Parakeets	Size of spleen, mm	Isolation of virus*		Parakeets	Size of spleen, mm	Isolation of virus*	
		Result	From			Result	From			Result	From
Females				Females				Females			
1	1.5×1.5	+	KI	11	2×2	−		21	2×2	−	
2	5×7	+	KI	12	3×3	−		22	3×3	−	
				13	4×4	−		23	5×5	−	
Males				14	4×3	+	KI	24	3×3	+	KI
3	1×1	−		15	10×10	+	KI, SL	25	2×2	+	SL
4	2×2	−						26	4×4	+	KI, SL
5	3×2	−		Males				27	5×5	+	KI, SL
6	3×2.5	−		16	2×2	−		28	6×6.5	+	KI, SL
7	3×3	−		17	2×2	−		29	12×8	+	KI, SL
8	2×2	+	KI	18	5×5	−		Males			
9	8×6	+	SL	19	2×2	+	SL	30	1×1	−	
10	6×7	+	KI, SL	20	2×2	+	KI	31	2×2	−	
								32	1×1	+	SL
								33	2×2	+	SL
								34	3×3	+	SL
								35	3×3	+	SL
								36	2.5×2.5	+	KI, SL
								37	5×4	+	KI, SL
Positive: 5 (50%)				Positive: 4 (40%)				Positive: 12 (70.5%)			

* KI = pooled kidney and intestine material; SL = pooled spleen and liver material.

RESULTS

Experience gained from the autopsies of many thousands of parakeets has indicated that if the spleen has a diameter of 4 or 5 mm or more, the viral agent is usually readily recovered by subinoculation of mice with suspensions of the organs of the infected bird. Exceptions have, however, been encountered. At least in the early stages of the acute and some of the more chronic infections, even birds with relatively small spleens may harbor virus in the liver or kidneys or cloacal contents. Therefore, mouse inoculation is essential to demonstrate the presence or absence of the viral agent.

As is shown in table 1, 12 of 17 infected, untreated parakeets (71%) still harbored the virus, as did 5 of 10 (50%) of the penicillin-treated birds and 4 of 10 (40%) of the aureomycin-treated group. These figures do not reflect a statistically significant reduction of the carrier rate among the treated flock, as compared with the untreated.

DISCUSSION

The experiments convincingly conform to previous observations which had shown that experimentally infected mice and ricebirds may appear to be successfully treated with penicillin (Meyer et al, 1947) provided treatment is instituted early in the infection and the antibiotic is used in large doses, but carriers do develop. Some ricebirds relapsed and died 22 to 27 days after discontinuation of intensive treatment with 6,000,000 units given in 17 consecutive days. It was then fully recog-

nized that penicillin merely suppresses the multiplication of the infective agent. Arbitrary termination of the treatment on the 17th day revealed that the drug had failed to sterilize the tissues, and, since the immunity mechanism is rather sluggish in ricebirds, the antibody level was inadequate to take over the important function of controlling the growth of the psittacosis agent. Apparently the same suppressive mechanism operates when aureomycin is administered to parakeets. The parenteral administration of 8 mg of aureomycin to a 30 g parakeet in 10 days constitutes, by comparison on a weight basis with other recently reported studies in man, a very large dose. Indeed, it is most unlikely that additional injections would be well tolerated by the birds. Since in the earlier experiments on mice and pigeons penicillin given in large doses over a long period failed to free the tissues from the psittacosis agent (Meyer et al, 1947), and since aureomycin appears to act on the psittacosis-lymphogranuloma infection in parakeet carriers in a manner similar to penicillin, it was deemed unnecessary to conduct further tests with this new antibiotic.

After completion of the studies, the report by Wagner (1949) fully supported the findings here recorded. He described experiments on mice treated with aureomycin after intracranial inoculation with psittacosis strains. Although 0.5 mg or less aureomycin prevented illness and death for two or more weeks following cessation of treatment, and in some cases indefinitely, the brain tissue of such mice continued to harbor the virus. In one case, the viral agent was isolated from brain tissue of a♂ mouse that survived 48 days and was then sacrificed. These results tend to indicate that aureomycin, as penicillin, is an impotent agent for treatment of psittacosis carriers. Nevertheless, it seems to be useful, in addition to penicillin, in the treatment of clinical infections (Brainerd et al, 1949). Furthermore, the results obtained by Loosli from use of the drug in mouse pneumonitis are indicative that aureomycin is to be favored over penicillin.

SUMMARY

Experimental carriers of psittacosis were developed by injecting parakeets with virus-infected embryonated yolk-sac suspensions. After a carrier stage was attained, some of these birds were treated with aureomycin and some with penicillin. The virus carrier rate in these treated groups was statistically undifferentiated from that in an untreated control group of birds 25 to 29 days after treatment was concluded.

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THE ACTION OF *d*-TUBOCURARINE CHLORIDE AND OTHER CURARIZING DRUGS ON THE GROWTH OF CERTAIN MICROORGANISMS

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Recent interest in *d*-tubocurarine and other curarizing drugs prompted investigation concerning the activity of these compounds on certain microorganisms. A review of the literature produced only some indirect references to this type of activity as stated by McIntyre (1947) except in the case of quinine (Johnson and Lewin, 1946), which is similar to the cinchona derivatives used in this study.

It is the purpose of this paper to present data concerning the sensitivity of various microorganisms to these compounds as determined by a paper disk and/or a tube method. The selection of the compounds was determined primarily by the availability. Most of them were prepared by one of us (DFM). The limited quantities also restricted the number of microorganisms tested or prevented complete testing of organisms selected.

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MATERIALS AND METHODS

A list of the compounds used will be found in table 1. References are given which will provide information concerning the chemical and pharmacological data for several of the compounds. Such data concerning the other drugs will be published soon by one of us (DFM). The compounds are numbered consecutively and these numbers will be used in place of the specific name throughout the remainder of the publication.

Aqueous 1 and 0.3% solutions of the compounds were made in distilled water. Aqueous 3% solutions were also made, except for compounds 1, 2, 3, 13, 15, 16, 17, 18, 19, and 22, in which cases 20% ethanol was the solvent.

The first method used to test the effects of these three different concentrations of the compounds on selected microorganisms was a paper disk method similar to that of Vincent and Vincent (1944). Four plates were prepared by pipetting 0.1 ml of an 18 to 24-hour broth culture of the test organism into a petri dish. The cultures contained approximately 300 million organisms per ml, as determined by comparison to nephelo-

TABLE 1.—*Compounds used in this study.*

1	*d*-tubocurarine chloride*	King (1948)
2	*d*-N-methyl-chondrodendrine iodide	Marsh et al (1948)
3	*p*-diethylaminodiphenylmethiodide	Prager and Jacobson (1936)
4	Trimethylammonium-p-toluidineiodide	Prager and Jacobson (1936)
5	Diethyl-m-toluidinemethiodide	To be published
6	*p*-diethylaminochlorobenzene methiodide	To be published
7	*p*-trimethylammoniumbromobenzene iodide	Prager and Jacobson (1936)
8	Phenyltrimethylammonium iodide	Prager and Jacobson (1936)
9	*l*-naphthylmethylpiperidine methiodide	To be published
10	*l*-naphthylmethylmorpholine methiodide	To be published
11	*l*-naphthylmethyldiethylamine methiodide	To be published
12	Quinine methiodide	Marsh and Pelletier (1948)
13	Cinchonine methiodide	Marsh and Pelletier (1948)
14	Cinchonidine methiodide	Marsh and Pelletier (1948)
15	Quinineethiodide	Marsh and Pelletier (1948)
16	Quinineisoamylbromide	Marsh and Pelletier (1948)
17	Quinidinemethiodide	Marsh and Pelletier (1948)
18	Ethoxyethylmorpholinemethiodide	Lee et al (1936)
19	N-(3-phenylpropyl)-piperidine methiodide	To be published
20	N-(3-phenylpropyl)-morpholine methiodide	To be published
21	N-(3-phenylpropyl)-trimethylammonium iodide	Prager and Jacobson (1936)
22	N-methyl-oxyacanthine iodide	Marsh et al (1949)
23	N-methyl-oxyacanthine bromide	Marsh et al (1949)
24	Oxyacanthine sulfate†	Kondo and Tomita (1930)

* Supplied in part by Abbott Laboratories.
† This compound has no curariform activity.

metric standards. In the case of *Mycobacterium phlei* and *Myco. smegmatis*, it was necessary to use 0.2 ml of a 42 to 48-hour broth culture. Fifteen ml of the medium were added to the plate, and the plate was then rotated to facilitate mixing. The medium used was Difco nutrient agar, except for the use of Difco North's gelatin agar for *Vibrio metschnikovii*, *Sporotrichum schenckii*, and *Myco. smegmatis*, and Difco North's gelatin agar plus 3% glycerine for *Myco. phlei*.

After the medium had hardened, a control disk (Fisher Scientific Co., Item No. 9–897) was centrally placed on the plate and 0.2 ml of the solvent alone was immediately added. Three disks were singly placed at equal distances around this one and immediately inoculated with 0.2 ml of the test solution. It was necessary to add the solution immediately after the disks were placed on the agar. Otherwise they would absorb moisture from the medium, and when 0.2 ml of solution was added, it would flood the area around the disk. After the test solutions were added to the disks the plates were incubated at 37 C in an upright position.

After 24 hours of incubation, the plates were examined and the width of the inhibition zone, when present, was measured with a millimeter ruler and recorded. *Myco. phlei* and *Myco. smegmatis* required 48 hours of incubation before distinct zones of inhibition could be seen.

The recorded measurement of inhibition is the distance in millimeters from the edge of the disk to the edge of the inhibition ring. The absence of an inhibition zone from around the outside of the disk was recorded as O. Thus, action directly under the disk was disregarded since it could not be ascribed to the effect of the drugs alone. In all instances triplicate plates were prepared for each dilution of each compound. The readings given are the averages of the three readings taken for a partcular concentration of the compound tested.

A number of microorganisms were first tested with compound 1 (*d*-tubocurarine chloride), compound 2 (*d*-N-methyl-chondrodendrine iodide), and compound 12 (quinine methiodide). Using these results, shown in table 2, as a basis, representative organisms were selected and tested against the remaining compounds, using both the disk and broth method. The broth method is described below.

TABLE 2.—*The action of compounds 1, 2, and 12 on certain microorganisms.*

Organism	Compound 1			Compound 2			Compound 12		
	Concentration of compound								
	3%	1%	0.3%	3%	1%	0.3%	3%	1%	0.3%
	Disk Method (values in millimeters)								
B. megatherium	6	2	0	1	0	0	8	2	0
Sar. lutea ATCC 272	9	8	5	7	3	2	8	2	1
Staph. aureus	2	0	0	2	0	0	4	1	0
Alcaligenes faecalis	0	0	0	0	0	0	0	0	0
Neisseria catarrhalis	4	0	0	2	0	0	4	0	0
Proteus vulgaris	0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0	0
S. enteritidis	0	0	0	0	0	0	0	0	0
S. paratyphi	0	0	0	0	0	0	0	0	0
S. schottmuelleri	0	0	0	0	0	0	0	0	0
	Concentration of compound								
	0.18%	0.06%	0.018%	0.18%	0.06%	0.018%	0.06%	0.06%	0.018%
	Broth Method								
Staph. aureus		3+	5+					5+	5+
Alcaligenes faecalis				5+	5+	5+	5+	5+	5+
S. enteritidis		5+	5+	4+	5+	5+	5+	5+	5+
S. paratyphi		5+	5+	5+	5+	5+	5+	5+	5+
S. schottmuelleri		5+	5+	4+	5+	5+	4+	5+	5+

Values in millimeters are width of the zone of inhibition.
Aqueous and ethanol controls were 5+ in all cases.
There was some indication that the *d*- and *l*- forms of these compounds might act differently, and as the two forms were available for compound 2, they were tested with three organisms as recorded in table 3.

The second method used to determine the action of these compounds on the microorganisms involved the use of a broth medium. Test tubes of uniform diameter were selected, and 4.7 ml of Difco nutrient broth were dispensed into them. The tubes of broth were then stoppered and autoclaved. During autoclaving, 0.1 ml of the broth was lost, leaving 4.6 ml of broth in the tube. The tubes were placed in racks, in groups of three, and 0.3 ml of a test solution was added to each of the three tubes, providing triplicate tubes for each dilution. A 24-hour broth culture containing approximately 300 million organisms per ml was then added in 0.1 ml amounts to each of the tubes. It was again necessary to use 48-hour broth cultures of *Myco. phlei* and *Myco. smeg-*

matis. The tubes were then incubated at 37 C for 22 to 24 hours, after which time readings were made. *Myco. phlei* and *Myco. smegmatis* cultures were read after 46 to 48 hours of incubation. Difco thioglycollate broth was used for *Streptococcus pyogenes* and *Diplococcus pneumoniae*. Triplicate control tubes were also run for each organism, and in the case of the compounds dissolved in ethanol, 0.3 ml of 20% ethanol was added to each control tube.

Several compounds, including all of those which produced noticeable inhibition of *Myco. phlei* or *Myco. smegmatis*, were tested against *Myco. tuberculosis* (bovine) ATCC No. 9805 and *Myco. tuberculosis* (human) Youmans HRv 37 strain. The method of testing was the same in principle as described above, but the procedure varied slightly. Difco tuberculosis (Tb) broth was dispensed into tubes in 4.3 ml amounts and autoclaved. After cooling, 0.4 ml of sterile Dubos Tb medium serum was added to each tube of broth. The final amount of broth in each tube was then 4.6 ml. After addition of 0.3 ml of the test solution the tubes were incubated 20 to 22 hours in order to detect possible contamination. Each tube was then inoculated with 0.1 ml of the test organism from a 6-week-old broth culture.

After inoculation, the cotton plugs were cut just above the lip of the tube, burned, and shoved below the lip of the tube with a grooved cork, which was also burned before insertion. The corked and plugged tubes were then incubated at 37 C for 24 days. Control tubes were prepared by using the same technique. Controls for the compounds in ethanol solution contained in addition 0.3 ml of 20% ethanol.

The final concentrations of the 3, 1, and 0.3% solutions of the compounds in the broth are respectively: 0.18, 0.06, and 0.018%. The final concentration in broth of 20% ethanol used in the controls is 1.2%.

All readings were made by a visual comparison of growth in the test solution to growth in control tubes. The recorded results are the averages of the three readings taken for a particular organism in a concentration of the compound tested.

Since 20% ethanol itself decreased the growth of some of the organisms in the broth medium, a 5+ system of recording growth was used. This was done as it seemed to provide a better method for recording the differences that occurred between certain aqueous and ethanol controls, and it also gave more latitude in recording degrees of inhibition of growth. Growth in the aqueous control tubes was called 5+.

RESULTS

The results are tabulated in tables 4, 5, 6, and 7. It will be noted that, in general, the inhibitory effects of the compounds correlated well by the two methods of testing.

TABLE 3.—*Comparing the action of* d- *and* l- *forms of N-methyl-chondrodendrine iodide on three organisms by the disk method.*

Organism	Concentration of *d*-form			Concentration of *l*-form		
	3%	1%	0.3%	3%	1%	0.3%
B. anthracis	3 mm*	0	0	6 mm	0	0
B. megatherium	5 mm	0	0	6 mm	2 mm	0
B. subtilis	3 mm	0	0	3 mm	0	0

* Values in millimeters are width of zone of inhibition.

The gram-positive organisms were the most susceptible to the action of the compounds. Coccus and bacillus forms did not always react similarly in degree of sensitivity to a particular drug. Of the gram-positive organisms tested by both methods, *Staphylococcus albus* was the least sensitive and *Sarcina lutea* (W. Va) the most sensitive to the action of the compounds.

By the disk method, *Bacillus anthracis* was inhibited by the 3% concentration of every compound except 10 and 15. The amount of inhibition was sometimes small, however. Compounds 3, 16, and 18 gave the largest inhibition zones of the 3% concentrations. The inhibition zones produced by the use of those compounds were often of the multiple ring type, consisting of three to five rings of inhibition. This multiple ring type of inhibition was encountered only with the spore-forming organisms and was believed to be associated with

TABLE 4.—*The action of certain curarizing drugs on certain gram-positive organisms as determined by the paper disk method.*

Organism	Concentration of compound	Compounds*																						
		1	2	3	4	5	6	7‡	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
B. anthracis	3.0%	4*	3†	9	1.5	1	2.5		2.5	P	P	5	8	5	1.5	0	7.5	1	5.5	4	1.5	2.5	4	P	P
	1.0%	0	0	5	0	0	0	3	0	P	P	0	3	0	0	0	6.5	0	3.5	0	0	0	2.5	P	P
	0.3%	0	0	2.5	0	0	0	0	0	9§	0	0	0	0	0	0	4.5	0	1.5	0	0	0	0	10§	3
B. subtilis	3.0%	2	3†	5	2.5	0	1		0	P	P	0	6	4	1	1.5	7	1	1.5	0	1.5	0	4	P	P
	1.0%	1	0	4.5	0	0	0	0	0	P	P	0	3	1.5	0	0	5	0	0	0	0	0	0	P	P
	0.3%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4.5	0	0	0	0	0	0	10§	6
G. tetragena	3.0%	4	6	17.5	5.5	4.5	8.5		2.5	P	P	5	4	0	8	7	14	7	4	8.5	4.5	11	12	P	P
	1.0%	2	4	13	1	0	3	1	0	P	P	0	2	0	2.5	5	11	4	3	5.5	0	3.5	9	P	P
	0.3%	0	0	11.5	0	0	4.5	0	0	0	0	0	0	0	0	0	8	0	1	0	0	0	2	0	1
S. lutea (W. Va.)	3.0%	12	11	11	15	13	4.5		9	P	P	8.5	14	15	11	6.5	14	10.5	3.5	12.5	2.5	13.5	10	P	P
	1.0%	9	8	9.5	13	10	0	7	4	P	P	4.5	10	10	7	2.5	15	9	1.5	9.5	0	8	5.5	P	P
	0.3%	6	4	3.5	7.5	5	0	2	0	11	3	0	6	9	0	0	12.5	5.5	0	3	0	0	2	9	7
Staph. albus	3.0%	3	4	12	4.5	3	4.5		1.5	P	P	3.5	5	7	2	0	10.5	1	4	6	0	3	7.5	P	P
	1.0%	2	0	10.5	1	0	0	0	0	P	P	0	3	2	0	0	7	0	2	1	0	0	5	P	P
	0.3%	0	0	7	0	0	0	0	0	0	0	0	0	1	0	0	5.5	0	0	0	0	0	1.5	1.5	4.5

P—Compound formed a precipitate. * All values given are in millimeters and represent the width of the zone of inhibition.
† *l*-form of the compound. ‡ Insolubility prevented preparation of 3% concentration. § Double ring zones of inhibition.

TABLE 5.—*The action of certain curarizing drugs on certain gram-positive organisms as determined by the broth method.*

Organism	Controls	Concentration of compound	Compounds																					
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
B. anthracis	Aqueous 5+ ethanol 4+	0.18%	2+*	0*†	0	4+	3+	5+		3+	?	?	5+	2+*	2+*	5+	4+*	0	4+*	2+*	4+*	3+	5+	+*
		0.06%	5+	5+	0	5+	4+	5+	5+	4+	3+	0	5+	3+	5+	5+	5+	0	5+	5+	5+	4+	5+	2+
		0.018%	5+	5+*†	4+	5+	5+	5+	5+‡	5+	4+	5+	5+	3+	5+	5+	5+	+	5+	5+	5+	5+	5+	5+
D. pneumoniae	Aqueous 5+ ethanol 4+	0.18%	+*	4+*†	0*	0	0	0		0	?	?	0	+	+*	+	0	0	2+*		0	0	5+	0
		0.06%	5+	6+	+	+	0	0	3+	2+	0	3+	2+	5+	5+	−	+	0	5+		+	0	5+	3+
		0.018%	5+	4+	+	4+	+	+	5+‡	4+	+	3+	5+	5+	5+	5+	2+	+	5+		2+	2+	5+	5+
G. tetragena	Aqueous and ethanol 5+	0.18%	2+	+*†	0	2+	+	⁘		3+	?	?	3+	0	5+	+	0	0	2+	5+	+	+	0	0
		0.06%	+	2+	0	3+	2+	3+	4+	4+	+	3+	5+	6+	5+		+	0	3+	5+	+	3+	2+	+
		0.018%	5+	4+†	+	4+	4+	5+	5+‡	4+	3+	5+	5+	7+	5+	5+	2+	+	6+	5+	5+	5+	5+	4+
S. lutea (W. Va.)	Aqueous and ethanol 5+	0.18%	+	0†	0	+	0	0		0	?	?	+	0	+	+	+	0	+	2+	+	+	0	0
		0.06%	+	0	0	+	0	2+	0	3+	+	0	3+	2+	0		2+	0	2+	3+	+	2+	+	+
		0.018%	+	+†	0	3+	3+	3+	3+‡	5+	3+	2+	4+	5+	3+	3+	5+	+	5+	5+	2+	3+	3+	+
Staph. albus	Aqueous and ethanol 5+	0.18%	2+	0†	0	3+	3+	3+		4+	?	?	4+	2+	3+	2+	+	0	3+	5+	+	5+	5+	0
		0.06%	0	0	0	5+	5+	5+	5+	5+	3+	3+	5+	3+	5+		5+	+	5+	5+	4+	5+	5+	0
		0.018%	5+	3+†	+	5+	5+	5+	5+	5+	5+	4+	5+	5+	5+	4+	5+	2+	5+	5+	5+	5+	5+	5+
Str. pyogenes	Aqueous and ethanol 5+	0.18%	3+	5+	+	2+	2+	2+		5+	?	?	+	3+	3+		4+	+	5+		3+	5+	5+	3+
		0.06%	5+	5+	+	3+	5+	5+	4+	5+	3+	+	3+	5+	5+		3+	+	5+		5+	5+	5+	5+
		0.018%	5+	5+	2+	5+	5+	5+	5+‡	5+	4+	3+	5+	5+	6+		5+	+	5+		5+	5+	5+	5+

0—No visible growth in the broth tubes. ?—Cloudiness in broth prevented positive determination of no visible growth.
* To be compared to the ethanol control. † *l*-form instead of *d*-form of this compound. ‡ An 0.00% concentration instead of 0.018%.

TABLE 6.—*The action of certain curarizing drugs on certain gram-negative bacteria, acid fast bacteria and fungi as determined by the paper disk method.*

Organism	Concentration of compound	Compounds*																							
		1	2	3	4	5	6	7†	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Esch. coli	3.0%	0*	0	6	0	3	3.5		0	P	P	1.5	3	2	4	0	4	0	0	2	0	1.5	0	P	P
	1.0%	0	0	2.5	0	0	0	0	0	P	P	0	0	0	0	0	0	0	0	1	0	0	0	P	P
	0.3%	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	P	P
K. pneumaniae	3.0%	6	0	8.5	0	2	2.5		0	P	P	3	7	0	0	0	4	0	0	5.5	0	0	4	P	P
	1.0%	1	0	4.5	0	0	0	0	0	P	P	0	2	0	0	0	2	0	0	1	0	0	0	P	P
	0.3%	0	0	2.5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2
S. typhosa	3.0%	0	0	9	0	3	3.5		0	P	P	0	0	2	0	0	4	0	0	4.5	3	0	0	P	P
	1.0%	0	0	9	0	0	0	0	0	P	P	0	0	0	0	0	1.5	0	0	2	0	0	0	P	P
	0.3%	0	0	1.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V. metschnikovii	3.0%	0	0	8	0	0	2		0	P	P	0	0	0	0	0	0	0	0	0	0	0	0	9.5	P
	1.0%	0	0	5	0	0	0	0	0	P	P	0	0	0	0	0	0	0	0	0	0	0	0	6.5	P
	0.3%	0	0	3	0	0	0	0	0	3.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Myco. phlei	3.0%	11	5	16.5	2.5	0	3.5		0	11.5	9	6	0	0	0	13	14.5	2	0	0	0	0	8	0	P
	1.0%	5	0	14.5	0	0	0	0	0	8.5	8	2.5	0	0	0	1.5	10	0	0	0	0	0	0	0	P
	0.3%	0	0	10	0	0	0	0	0	0	2	3.5	0	0	0	0	5	0	0	0	0	0	0	0	0
Myco. smegmatis	3.0%	7	7	14.5	0	0	0		0	14.5	10	6.5	0	0	0	0	13	0	0	0	0	0	4	5	P
	1.0%	4	2	10	0	0	0	0	0	9	10	0	0	0	0	0	12	0	0	0	0	0	0	0	P
	0.3%	0	0	4.5	0	0	0	0	0	5	5	0	0	0	0	0	5	0	0	0	0	0	0	0	2.5
C. albicans	3.0%	0	0	14	12	9.5	2.5		7	P	P	6.5	0	1	0	0	6.5	0	0	7	1.5	12.5	7	P	P
	1.0%	0	0	7	8	7	0	3	3	P	P	2	0	0	0	0	5.5	0	0	5.5	0	6.5	3	P	P
	0.3%	0	0	6	0	0	0	0	0	9	2.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. schenckii	3.0%	0	0	15.5	0	4	6.5		0	16	13	16	9	0	0	6.5	14	7	0	3.5	4	15	0	P	P
	1.0%	0	0	10	0	0	0	0	0	11	10	12	2	0	0	1.5	14	1.5	0	0	0	10	0	P	P
	0.3%	0	0	4.5	0	0	0	0	0	7.5	8	2	0	0	0	0	6.5	0	0	0	0	3	0	0	0

P—Compound formed a precipitate.
* All values given are in millimeters and represent the width of the zone of inhibition.
† Insolubility of this compound prevented the preparation of a 3% solution.

TABLE 7.—*The action of certain curarizing drugs on certain gram-negative, acid-fast bacteria and fungi as determined by the broth method.*

Organism	Controls	Concentration of compound	Compounds																					
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
K. pneumoniae	Aqueous 5+	0.18 %	+*	3+*†	0	2+	3+	3+		5+	?	?	+	+*	2+*	3+	2+*	+*	3+*	4+*	5+	5+	4+	2+*
	ethanol 4+	0.06 %	3+	5+	2+	3+	4+	4+	5+	5+	4+	+	2+	2+	5+		3+	2+	5+	5+	2+	5+	5+	2+
		0.018%	5+	5+	4+	5+	5+	5+	5+	5+	5+	5+	4+	3+	5+	4+	4+	5+	5+	5+	3+	5+	5+	3+
S. typhosa	Aqueous and	0.18 %	3+	5+†	0	5+	3+	4+		5+	?	?	+	2+	5+	5+	2+	+	4+	4+	3+	4+	5+	5+
	ethanol 5+	0.06 %	4+	5+	0	5+	5+	5+	5+	5+	0	0	3+	3+	5+		3+	2+	5+	5+	4+	5+	5+	5+
		0.018%	5+	5+†	2+	5+	5+	5+	5+	5+	0	2+	4+	5+	5+	5+	5+	5+	5+	5+	5+	5+	5+	5+
Myco. phlei	Aqueous and	0.18 %	+	0*	0	+	2+	+		5+	?	?	0	5+	5+	5+	2+	+	2+	3+	5+	5+	5+	0
	ethanol 5+	0.06 %	0	0	0	4+	4+	2+	4+	5+	+	2+	3+	3+	5+		5+	+	5+	4+	5+	5+	5+	0
		0.018%	3+	3+†	+	5+	4+	6+	5+	5+	5+	5+	4+	2+	5+	5+	5+	2+	5+	5+	5+	5+	5+	2+
Myco. smegmatis	Aqueous and	0.18 %	+	0†	0	5+	3+	+		5+	?	?	2+	5+	3+	5+	2+	0	3+	3+	4+	4+	5+	0
	ethanol 5+	0.06 %	+	2+	0	5+	5+	3+	5+	5+	0	0	3+	5+	5+		3+	+	5+	4+	5+	5+	5+	+
		0.018%	3+	5+†	+	5+	5+	5+	5+	5+	2+	2+	4+	5+	6+	5+	5+	2+	5+	5+	5+	5+	5+	2+
Myco. tuberculosis (human)	Aqueous 5+	0.18 %	+*	+	+*			5+			+		+				2+*	3+*	4+*					2+*
	ethanol 4+	0.06 %	+	3+	3+			7+			+		3+				4+	5+	5+					5+
		0.018%			5+			7+			2+		5+				5+		5+					5+
Myco. tuberculosis (bovine)	Aqueous and	0.18 %	+	+	+			7+			3+	0	5+	7+			5+	0	7+					3+
	ethanol 5+	0.06 %	2+	2+	6+			7+			5+	5+	5+	6+			5+	+	6+					5+
		0.018%			5+			5+			5+	6+	5+	5+			5+	3+	5+					5+
C. albicans	Aqueous 5+	0.18 %	3+*	0*†	0	5+	0	0		0	?	?	0	4+*	+	2+	6+*	+*	2+*	6+*	0	6+	0	0
	ethanol 6+	0.06 %	4+	+	+	6+	+	+	+	2+	0	0	+	6+	4+		5+	+	5+	4+	2+	7+	+	+
		0.018%	5+	6+	+	7+	2+	5+	6+	5+	3+	+	4+	7+	5+	4+	7+	2+	6+	6+	3+	8+	5+	6+

See footnotes in table 5.

the presence of the spores. *B. subtilis* was not inhibited by compounds 5, 7, 8, 11, 19, and 21. Also in many instances when inhibition occurred the zones were smaller with *B. subtilis* than with *B. anthracis*. In broth *B. anthracis* was completely inhibited by 0.18 and 0.06% concentrations of compounds 3, 10, and 16. All of these gave rather large inhibition zones by the disk method.

In the case of compound 2 (N-methyl-chondrodendrine iodide) we used both the *d*- and *l*- forms, as will be noted in the various tables. In table 3 the three concentrations of each form were used with *B. anthracis*, *B. megatherium*, and *B. subtilis*. It is to be noted that the *l*- form produced the larger zones in the 3% solutions and even some inhibition in the 1% concentration. This and the other data indicate there would probably be differences in these forms among the other compounds, and at least in this one instance the *l*- form is more active than the *d*- form.

D. pneumoniae was completely or partially inhibited in broth by the 0.18% concentration of all the compounds except 21. The 0.06% concentration of compounds 5, 6, 9, 16, and 20 caused complete inhibition, and several others produced a reduced growth rate. The 0.018% solutions of some compounds reduced growth, but there was no instance of complete inhibition of growth. *D. pneumoniae* seemed to be stimulated by 0.06% of the *d*- form of compound 2.

Gaffkya tetragena was inhibited in the disk method to some degree by all compounds except 13 and also in the broth method except by compounds 13 and 18. Growth of *G. tetragena* seemed to be stimulated by 0.06 and 0.018% concentrations of compound 12 and by 0.018% of compound 17.

Three percent solutions of all compounds inhibited *Sarcina lutea* producing in some instances the largest zones obtained with any of the organisms tested. Compounds 1 and 12 were able to cause inhibition of this organism in concentrations as low as 0.1%. Because of the marked sensitivity of *Sar. lutea* for these compounds it might be possible to use it as a test organism to determine the presence of these drugs in various types of solutions.

Staph. albus was inhibited on agar by 3, 1, and 0.3% of compounds 3, 16, and 22. These three compounds were also the most active in broth at 0.18, 0.06, and 0.018% concentrations. It is to be noted that 0.06% of compound 1 completely inhibited this organism, while at the 0.18% concentration of this compound the organism grew, though definitely inhibited.

Str. pyogenes was not adequately tested by the disk method, so only the results of the broth method will be included. Compound 10 was the most active inhibitor although, of the compounds tested, all except 8, 17, 20, and 21 reduced the growth in some or all of the concentrations.

On the gram-positive organisms as a group, compound 3 (*p*-diethylaminodiphenylmethiodide) and compound 16 (quinineisoamylbromide) were the most active inhibitors of the growth of these organisms.

The gram-negative bacteria were more resistant to the compounds than were the gram-positive organisms. Again compound 3 was the most active. By the disk method, 3, 1, and 0.3% concentrations of this compound inhibited *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhosa*, and *V. metschnikovii*. Various other compounds in 3% solutions inhibited the gram-negative organisms. However, the rings of inhibition produced by all the other compounds were smaller against all

gram-negative organisms than those produced by compound 3. In broth, only compounds 3, 9, and 10 caused complete inhibition of *K. pneumoniae* and *S. typhosa* in their 0.18% concentrations.

Myco. phlei and *Myco. smegmatis* were tested by the disk method with all compounds and by the broth method by all except compounds 23 and 24. Depending upon the activity and availability, certain of the compounds were used in the broth method with *Myco. tuberculosis* (human) and *Myco. tuberculosis* (bovine). With regard to *Myco. phlei* and *Myco. smegmatis*, compounds 1, 2, 3, 9, 10, 11, 13, 16, and 22 were the most active, producing varying degrees of inhibition.

Myco. tuberculosis (human) was not completely inhibited by any of the compounds although marked inhibition was noted with 0.18% of 1, 2, 3, 9, and 11. The 0.06% concentrations of 1 and 9 also produced marked inhibition. Compounds 1 and 9 were the most active inhibitors, whereas 6 produced increased growth as measured by visual comparison with the controls.

Myco. tuberculosis (bovine) was completely inhibited by 0.18% of compounds 10 and 16 and markedly inhibited by this concentration of compounds 1, 2, and 3. Compound 16 was the most inhibitory. However, increased growth was recorded in the case of compounds 3, 6, 10, 12, and 17, being greatest with 6, 12, and 17.

These results indicate that the action of these and similar compounds should be tested further, particularly with *Myco. tuberculosis* (human), and perhaps a suitable chemotherapeutic agent for tuberculosis may be found among them.

Candida albicans was inhibited in the disk method by several compounds but in the 0.3% concentration only by 3, 9, and 10. Compound 3 was the most active inhibitor. In the broths, the ethanol control showed some increase in growth as did certain concentrations of compounds 2, 4, 7, 12, 15, 20, and 22. The 0.18% solution of several compounds inhibited growth completely, as did the 0.06% concentration of 9 and 10. None of the 0.018% concentrations completely inhibited growth, and only 3 and 10 showed marked inhibition.

S. schenckii was tested only by the disk method, being completely inhibited by the 3, 1, and 0.3% solutions of compounds 3, 9, 10, 11, 16, and 21. Some of the other drugs reduced growth of this organism in the 3% and/or 1.0% concentrations.

Compound 3 (*p*-diethylaminodiphenylmethiodide) had by far the greatest inhibitory action of the compounds tested. It inhibited every organism tested by either method to some degree. Compound 16 (quinineisoamylbromide) was only slightly less inhibitory than the one just mentioned. It inhibited to some degree every organism except *V. metschnikovii*, in its 3% concentration.

The ethanol solution had no inhibitory effect when used as a control in the disk method of testing. It did, however, have a small inhibitory effect in the broth, though the final concentration was only 1.2%. *K. pneumoniae*, *B. anthracis*, *D. pneumoniae*, and *Myco. tuberculosis* (human) were the organisms which were inhibited to some degree by the ethanol, whereas *C. albicans* showed an increased growth.

DISCUSSION

The correlation obtained by the two methods of testing was generally good, especially when one considers the difference in the physical nature of the two methods. On agar, 0.2 ml of a solution of a drug was placed on one

area, and though the amount of solution that diffused into the area probably varied with the size of the molecule, the evenness and size of the zones indicated rapid diffusion through the medium. In broth, it was possible to calculate the final concentration of the drugs, and diffusion was assumed to be equal throughout the medium.

Stimulation of the growth of the microorganisms could not be reliably determined on the solid medium. An indication of stimulation would be larger colonies, but only in the case of *C. albicans* did the colonies appear to be larger in the presence of certain of these compounds than colonies on the control plates. In broth it was possible to approximate the amount of stimulation in any particular concentration of a compound. Evidence of stimulation was noted particularly with *C. albicans* and *Mycobacterium*.

A comparison of the results of the two methods shows that an organism that was inhibited 9 mm by the disk method of testing was generally totally inhibited in broth. There was, however, variability within compounds and organisms.

All compounds, except 24 (oxyacanthine sulfate) are quaternary ammonium compounds. Oxyacanthine sulfate is also the only compound without curariform activity. The compounds that were most inhibitory, compound 3 (*p*-diethylaminodiphenylmethiodide) and compound 16 (quinineisoamylbromide) have little in common in structure other than the presence of quaternary nitrogen in both drugs.

Placing these compounds into groups on the basis of similarity of molecular structure (however, neither the formulas nor groupings are included in this publication) provided no information on determining the molecular arrangement which was most active, nor did the compounds within any groups react in the same manner.

There seems to be no correlation between curariform activity and ability to inhibit the growth of the microorganisms tested. Compound 3, the most active inhibitor in the series, has only low activity in the curarization on animals.

It is most likely that the *d*- and *l*-forms of the compounds, if available, would react differently. There is some supporting evidence for this with compound 2, in which case the *l*- form was more active than the *d*- form of N-methyl-chondrodendrine iodide.

The growth of *Myco. tuberculosis* (human) was definitely inhibited by the 0.18 and 0.06% concentration of several of the compounds. These results indicate that further investigation of the action of these or similar compounds on the *Mycobacterium* by both in vitro and in vivo methods is warranted.

As noted, *Sar. lutea* exhibited the greatest degree of susceptibility to all of these compounds. Two strains were tested by several of the compounds and the W. Va. strain was more sensitive than the ATCC 272. It might be possible to use this organism as a test organism to determine the presence or potency of these drugs.

The experiments were not designed to determine the mode of action of these alkaloids on the various microorganisms used.

SUMMARY

Certain quaternary ammonium compounds having curariform activity were tested by two methods in order to determine their action on the growth of certain microorganisms.

The compounds were found to cause inhibition of growth in many cases. The compound *p*-diethylaminodiphenyl methiodide caused some inhibition of

every organism tested, while another, quinineisoamylbromide, inhibited every organism but one, *Vibrio metschnikovii*. Practically all of the compounds tested inhibited to some degree, at least, all the gram-positive organisms. The gram-negative organisms were more resistant. However, a number of the compounds inhibited their growth, *p*-diethylaminodiphenylmethiodide and quinineisoamylbromide were again the most active.

Many of the compounds tested inhibited the growth of *Mycobacterium phlei* and *Myco. smegmatis*, and, of these compounds, five were capable of markedly inhibiting the growth of *Myco. tuberculosis* (bovine). Seven compounds markedly inhibited *Myco. tuberculosis* (human); these included the five which also inhibited the bovine strain. Quinineisoamylbromide was the most active against *Myco. tuberculosis* (bovine), while *d*- tubocurarine chloride and *l*-naphthylmethylpiperdine methiodide were the compounds most inhibitory to *Myco. tuberculosis* (human).

Many of the compounds also inhibited the growth of *Candida albicans* and *Sporotrichum schenckii*.

All compounds inhibited *Sarcina lutea*.

Some of the compounds caused a stimulation of growth of various species of *Mycobacterium*, particularly *Myco. tuberculosis* (bovine). The growth of *C. albicans* was stimulated by a greater number of the compounds.

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ON THE TOXIC AND ALLERGIC MANIFESTATIONS CAUSED BY THE TISSUES AND FLUIDS OF ASCARIS. II. EFFECT OF DIFFERENT CHEMICAL FRACTIONS ON WORM-FREE, INFECTED AND SENSITIZED GUINEA PIGS

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In a previous publication (Sprent, 1949), it was shown that 1% suspensions of the body fluid, intestine, cuticle, muscle and ovary of *Ascaris lumbricoides* from the pig, as well as the whole worm, failed to produce symptoms of shock when injected intravenously into mice, rabbits and guinea pigs, provided that no previous sensitization had occurred. The question as to whether ascaris contains, in its body, substances which are primarily toxic was discussed and it was concluded that the positive findings of various investigators who used animals which normally harbor nematodes must be discounted as evidence in this connection, in view of the possibility that the observed toxicity was due to hypersensitivity through infection. It was suggested that another possible cause of toxic effects might be the production of toxic substances consequent upon protein disintegration during manipulation of the worm substances.

The following experiments were designed firstly to test the effects of larger amounts of ascaris tissues and of different chemical fractions of whole worms and the body fluid on worm-free guinea pigs, and secondly to discover whether any change in the toxicity of such materials occurs with the lapse of time after they have been removed from the worms. Experiments which show the influence of infection with parasitic nematodes and the effect of previous sensitization on the susceptibility of guinea pigs to the toxic effects of ascaris extracts are also included.

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METHODS

The chemical extracts used in these experiments were obtained from whole worm pulp and from the body fluid of *A. lumbricoides* var. *suis*. The worms were obtained from the abattoir within two hours of the death of the host. Body fluid was removed by puncturing the lower end of suspended worms and collecting the fluid which dripped off them. Whole worm pulp was obtained by mincing living worms in the Waring Blendor for two minutes with an equal quantity of physiological saline. In the case of extracts A, this pulp was left overnight in the refrigerator and then centrifuged for ½ hour at 2000 r.p.m.; the supernatant fluid was used as the parent extract (extract A_1) from which other extracts were made. The subsequent treatment to which these fluids were subjected was directed towards the separation of various fractions of protein hydrolysis according to figure 1.

Three methods of protein precipitation were used: precipitation with an equal volume of 10% phosphotungstic or trichloracetic acid or full saturation with ammonium sulphate. After addition of the precipitant, the mixture was mechanically stirred for ½ hour, refrigerated for several hours and then centrifuged for ½ hour at 2000 r.p.m. A sample of the supernatant fluid was tested for complete precipitation of proteins, dialyzed within cellophane tubing for 2 to 4 days against running tap water at 5 C and against distilled water for 8 hours in the refrigerator. The material was tested for the presence of the pre-

cipitant, and if it was absent, the dialyzed material was either subjected to further precipitation (in the case of extract A_3) or frozen at −72 C and lyophilized. When alcohol or acetone were used for precipitation, equal parts of the material to be precipitated and the alcohol or acetone were cooled in the refrigerator, mixed together and left overnight in the refrigerator. The precipitate was removed by centrifugation and washed with alcohol or acetone; the alcohol or acetone was removed from the filtrate by evacuation at room temperature and the residual fluid frozen and lyophilized.

Extract A_1 was subjected to boiling for 10 minutes in a water bath and to digestion at 37 C for 24 hours with 1 g pepsin per 100 cc of extract. The digested extract A_1 gave no precipitate with trichloracetic acid, and digestion of protein was regarded as complete. Whole worm pulp was subjected to partial digestion with pepsin (18 hours), and the resultant material was fractionated as extracts D (see table 1).

Extracts B were prepared in the way described by Rocha e Silva and Grana (1946), trichloracetic acid being added directly to the pulped worms before centrifugation. The supernatant fluid after centrifugation, dialysis and neutralization was used for injection and designated extract B_2; part of this extract was lyophilized so that the dry weight of the material injected could be estimated. Extract B_2 corresponds to the extract E_0 of Rocha e Silva and Grana. Extract B_4 corresponds to their extract E_1 and consisted of the material precipitated by acetone after removal of "glycogen" from extract B_2 by precipitation with alcohol.

Extracts C were prepared in the same way as extracts A except that the worm pulp was left at room temperature instead of in the refrigerator for 18 hours. Table 1 is a list of the extracts made, showing the abbreviations employed in this paper and the approximate amounts in which they occur in 1 g of living worms. Several such analyses were made, and the amounts obtained varied according to the amount of original extract obtained from the worms in the first operation. This amount varied from 0.05 to 0.08 g per g worm in the case of A_1. Nitrogen determinations are also shown in table 1; they were performed according to the method of Zuazaga (1942).

Body fluid was treated in essentially the same way as extract A_1 except that ether extractable substances were removed by repeated shaking with ether and subsequent separation of the ether and aqueous fractions. The aqueous fraction was treated with trichloracetic acid, dialyzed and then precipitated with alcohol to remove polysaccharides. Four such analyses were made on

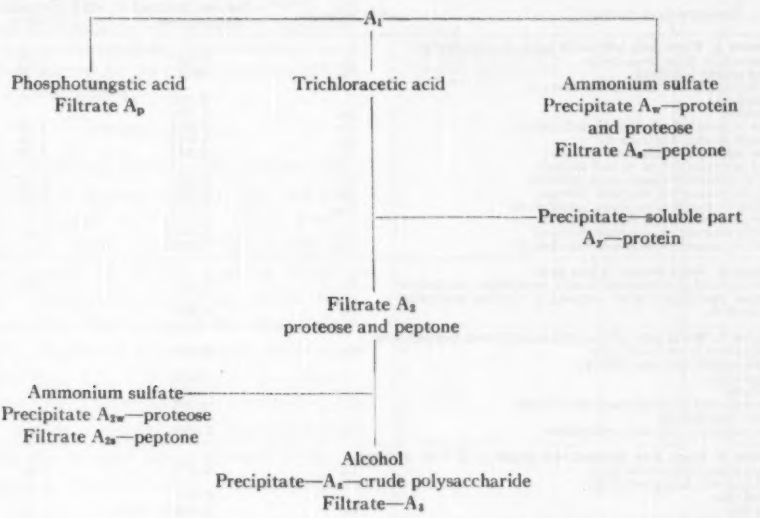

FIG. 1.—Treatment given parent extract in order to separate the various fractions of protein hydrolysis.

different samples of body fluid, and the amount (in grams) of each fraction per 100 cc of body fluid was found to be as follows: total solids after lyophilization, 5.6 g; ether extract, 1.6 to 1.9 g; material precipitated by trichloracetic acid, 1.7 to 2.4 g; crude polysaccharide, i.e., material precipitated by alcohol after removal of trichloracetic acid precipitate and dialysis, 0.1 to 0.2 g; "filtrate," i.e., residual substances after removal of alcohol precipitate and alcohol, 0.2 to 0.3 g.

Lyophilized preparations of the following materials were made by the method described in a previous publication (Sprent, 1949): ascaris cuticle, muscle, ovary, body fluid, intestine and metabolic products; whole worm preparations of *Moniezia expansa*, *Ascaris columnaris*, *Physaloptera maxillaris*, *Toxascaris* sp., *Ascaridia galli*, *Macracanthorhyncus hirudinaceus*, oligochaetes of the genera *Tubifex* and *Lumbricus* and a planorbid snail. In addition, various vertebrate tissues were lyophilized: normal guinea pig lung and kidney, the lungs of a guinea pig infected 8 days previously with 4000 ascaris eggs and the diaphragm of a guinea pig given 500 trichina larvae 28 days previously. These tissues were minced in the Waring Blendor until a uniform pulp was obtained and immediately frozen and lyophilized. Group A human erythrocytes and rabbit erythrocytes were obtained from whole blood by centrifugation and washing with physiological saline; they were frozen and lyophilized. Mr. J. Barlow of the Department of Biochemistry, University of Toronto, kindly supplied a lyophilized extract of bull's testicles, which was used as a source of hyaluronidase. The vitreous humour of cows' eyes was used as a source of hyaluronic acid and was injected in the fresh state after centrifugation. The pneumococcus polysaccharides were obtained from E. R. Squibb & Sons. One cc of combination A and B were mixed and injected, the mixture containing polysaccharides from types I through VIII and types XIV, XV and XVIII.

Before injection all solutions were neutralized to pH 7.0 and centrifuged to remove any particulate matter. Intravenous injections were made into the external saphenous vein in all cases. The guinea pigs designated "worm-free" in these experiments were all obtained from the Connaught Laboratory Farm of the University of Toronto. Every animal used was not examined

TABLE 1.—*Weights (in g) and nitrogen contents of different ascaris extracts per g of living worms.*

Description of extract	Abbreviation	Amount obtained per g worms	Nitrogen content (g/100 g)
Extracts A. Worm pulp left for 18 hours in refrigerator			
Supernatant from whole pulp	A_1	0.062	5.18
Supernatant (dialyzed)	A_2	0.037	12.20
Trichloracetic acid precipitate from A_1			
Insoluble		0.012	
Soluble	A_7	0.006	
After removal of precipitate and dialysis	A_3	0.018	1.70
Alcohol precipitate from A_3	A_4	0.016	1.96
After alcohol precipitation (filtrate)	A_5	0.003	3.39
After precipitation of A_1 and dialysis			
With phosphotungstic acid (filtrate)	A_p	0.006	0.00*
With ammonium sulphate (filtrate)	A_s	0.002	8.83
With ammonium sulphate (precipitate)	A_w	0.054	11.40
After precipitation of A_3 and dialysis			
With ammonium sulphate (filtrate)	A_{3s}	0.0017	1.96
With ammonium sulphate (precipitate)	A_{3w}	0.0002	
Extracts B. Fresh worms (whole pulp)			
After removal of trichloracetic acid precipitate and dialysis	B_3	0.04	0.92
Acetone precipitate after removal of alcohol precipitate from B_3	B_4	0.001	
Extracts C. Worm pulp left for 18 hours at room temperature			
Supernatant from whole pulp	C_1	0.070	
Trichloracetic acid precipitate			
Insoluble		0.007	
Soluble		0.005	
After removal of precipitate and dialysis	C_3	0.008	
Alcohol precipitate		0.002	
After removal of alcohol precipitate	C_4	0.005	
Extracts D. Worm pulp digested with pepsin at 37 C for 18 hours			
Supernatant from whole pulp	D_1	0.175	8.46
Trichloracetic acid precipitate			
Insoluble		0.005	
Soluble	D_7	0.002	
After removal of precipitate and dialysis	D_3	0.165	7.65
Alcohol precipitate		0.080	
After removal of alcohol precipitate	D_4	0.083	12.00

* Two preparations gave 0.00 and one gave 0.02 g %.

for parasites, but animals were picked at random throughout the course of the experiments so that 60 of the animals were examined in the following manner: The alimentary tract from esophagus to colon was removed and water was passed through it, the contents flowing into a sieve having 60 meshes to the inch. The alimentary tract was then opened along its length and passed between the jaws of a forceps, so that any material adherent to the wall passed into the sieve. The contents of the sieve were then washed and scraped into petri dishes for examination. In none of these 60 animals were any worms discovered. The guinea pigs which were infected with nematodes of the genus *Paraspidodera* were obtained from a different source. All the guinea pigs weighed between 350 and 500 g.

EXPERIMENTAL

Experiments on worm-free guinea pigs. Toxicity of ascaris tissues and extracts.

Experiment 1.—Body fluid freshly obtained was injected intravenously into groups of guinea pigs as follows: Four groups of 4 animals each were given 0.3, 0.4, 0.5 and 0.7 cc, respectively, 4 groups of 8 animals each were given 0.8, 1.0, 2.0 and 3.0 cc, respectively and 3 animals were given 4.0, 5.0 and 6.0 cc, respectively. The 3 animals given the largest doses showed dyspnea following the injection, but the others showed no symptoms.

Experiment 2.—Lyophilized body fluid was dissolved in water and 0.04, 0.13, 0.2, 0.25, 0.35, 0.4 and 0.5 g injected intravenously into 7 guinea pigs, respectively. One animal given 0.13 showed dyspnea following the injection; the remainder showed no symptoms.

Experiment 3.—Four different chemical fractions were prepared from fresh ascaris body fluid, and the respective proportions of these chemical fractions in body fluid were estimated (see Methods). Suspensions were made from these 4 fractions, i.e., protein (trichloracetic acid precipitate), crude polysaccharide, ether extract and "filtrate" (see Methods), in such a way that 1 cc of saline

TABLE 2.—*Effect following intravenous injection of different extracts (see table 1) on worm-free guinea pigs.*

Extract	Number of animals	Grams injected	Effect
A_1	1	0.03	−
A_1	4	0.05	−
A_1	9	0.10	+
A_1	2	0.15	+
A_1	5	0.20	++
A_1	2	0.25	++
A_1	3	0.30	++
A_1	5	0.40	+++ (3) ++ (2)
A_1	1	0.50	+++
A_1 (boiled)	2	0.25	+
A_1 (boiled)	1	0.40	+
A_1 (digested with pepsin)	4	0.25	+
A_1 (digested with pepsin)	2	0.50	+
A_2 (dialyzed A_1)	3	0.30	+++ (1) ++ (2)
A_7	2	0.05	++
A_7	2	0.10	++
A_7	2	0.20	++
A_8	2	0.05	+
A_8	3	0.10	+
A_8	3	0.20	+
A_8	1	0.30	+
A_8	1	0.10	−
A_8	1	0.20	−
A_8	1	0.25	−
A_8	1	0.03	−
A_8	1	0.05	−
A_8	7	0.10	+
A_8	1	0.25	+
A_9	2	0.07	−
A_9	2	0.05	−
A_9	2	0.02	−
A_9	2	0.02	−
A_W	3	0.25	+++
B_2	8	0.05	−
B_2	1	0.08	−
B_2	3	0.10	−
B_2	5	0.15	−
B_2	6	0.20	+
B_2	3	0.30	+
B_4	1	0.03	+
B_4	1	0.05	+
B_4	1	0.08	+
B_4	1	0.09	+
C_1	6	0.20	+++ (1) ++ (5)
C_1	2	0.30	+++ (1) ++ (1)
C_1	3	0.40	+++
C_1	2	0.04	+
C_1	1	0.06	++
C_1	5	0.20	++
C_1	9	0.10	+ (6) − (3)
C_1	2	0.15	+
C_1	1	0.25	+
D_1	3	0.20	++
D_1	1	0.40	+++
D_X (dialyzed D_1)	1	0.30	++
D_X	1	0.40	+++
D_7	1	0.04	−
D_7	1	0.10	−
D_7	1	0.30	++
D_8	2	0.10	++
D_8	2	0.20	+++
Ascaris cuticle	2	0.40	+++
Ascaris body fluid	1	0.40	−
Ascaris intestine	1	0.40	−
Ascaris muscle	1	0.40	++
Ascaris ovary	1	0.40	++
Lumbricus sp.	1	0.10	Collapse
Lumbricus sp.	1	0.20	Collapse and death
Lumbricus sp.	1	0.40	Collapse and death
Moniesia expansa	1	0.40	+++

+++ signifies death following symptoms of asphyxia, with marked pulmonary emphysema. ++ signifies severe dyspnea. + signifies slight dyspnea. − signifies no symptoms.

contained the separate fractions in the same proportions as each was present in 1 cc of body fluid (A). Other suspensions were made containing twice (B) and ten times (C) these amounts per cc. Four series of guinea pigs were used, one series for each chemical fraction. In each series there were 3 animals, which were injected with fractions A, B and C, respectively. All the animals injected with ether extract showed slight dyspnea, but with polysaccharide and protein there was slight dyspnea only in the case of injection B. "Filtrate" caused no symptoms following injections. None of the animals died as a result of the injection.

Experiment 4.—Fresh body fluid was allowed to stand at room temperature for 24 hours before injection. Five guinea pigs were injected intravenously with 3 cc each when the fluid was first removed from the worms, and no symptoms occurred. After 24 hours, 9 guinea pigs were injected with 2 cc and 3 guinea pigs with 3 cc intravenously. Six of the animals showed dyspnea and coughing, but none of them died.

Experiment 5.—Body fluid was kept in the refrigerator for 7 days and 3 cc was then injected intravenously into each of two guinea pigs. No symptoms followed.

Experiment 6.—The various whole worm extracts (see table 1) were injected intravenously into guinea pigs as shown in table 2. The amounts indicated were injected as a 10% solution in physiological saline.

From experiments 1 and 2, it was evident that large doses (approximately 1 g/kg body weight) of fresh ascaris body fluid can be injected intravenously into normal guinea pigs without causing death. Experiment 3 showed that none of the 4 fractions isolated from body fluid caused death in doses up to 10 times the amount calculated to be present in 1 cc of fresh body fluid. Experiments 4 and 5 showed that although body fluid which is refrigerated appears to remain non-toxic, body fluid which is allowed to remain at room temperature for 24 hours may cause dyspnea in normal guinea pigs. Experiment 6 (see tables 1 and 2) showed that the whole worm extract A_1 showed evidence of toxicity when 0.1 g was injected and that a dose of approximately 1 g/kg body weight caused death following symptoms which were very similar to those occurring in anaphylactic shock. The animals made very severe inspiratory efforts, collapsed and died within a few minutes of the injection; autopsy revealed marked pulmonary emphysema. The hyperexcitability which was observed to precede true anaphylaxis was, however, not shown in these animals. When proteins were precipitated by trichloracetic acid, phosphotungstic acid or ammonium sulphate, this toxicity was removed (see table 2). The toxic substance was not removed during dialysis, but it was mostly removed by boiling and filtering extract A_1, and also by peptic digestion for 24 hours. Partial digestion of the worm pulp with pepsin caused an increase in the toxicity of the nonprotein fraction. It was not possible to inject all the material precipitated by trichloracetic acid and ammonium sulphate from extract A_1 because it would not all dissolve in water or physiological saline. However, after dialysis of the whole precipitate and removal of the insoluble portion of the precipitate by centrifugation and filtration, the soluble portions, A_y and A_w, respectively, were found to cause the same effect as the original extract A_1. The material precipitated by alcohol caused no symptoms in a dose of 0.25 g, which is almost twice the amount of this material estimated to be present in 0.4 g of extract A_1. The re-

sults obtained with extract C (see tables 1 and 2) showed that there was some increase of toxicity, especially in the deproteinized extracts, as a result of decomposition at room temperature.

The injection of the supernatant fluid from different ascaris tissues after homogenization in saline showed that the cuticle was the only one to cause death in normal animals in doses up to 0.4 g. Injection of extracts of the cestode *Moniezia expansa* and an earthworm, *Lumbricus sp.*, which were prepared in the same way as ascaris extract A_1, showed that ascaris is not unique in inducing a toxic effect. The effect produced by the cestode was identical with that produced by ascaris, but the earthworm extract caused a different effect in that there appeared to be no respiratory involvement. Locomotor incoordination and spasmodic movements occurred very rapidly after injection, and were followed by collapse and death. Autopsy showed dilation of the right ventricle of the heart.

Experiments on guinea pigs infected with nematodes

Experiment 7.—Three guinea pigs were infected with 8000 eggs of *A. lumbricoides*. Ten days later two of these animals were injected with 0.5 cc of ascaris body fluid. Both of them showed acute anaphylaxis; one of them died within 5 minutes of the injection, and their lungs showed a considerable degree of emphysema. The third guinea pig, which received no injection of body fluid, showed no emphysema. Five similarly infected guinea pigs were injected in the same manner at 8, 10, 13, 17 and 19 days, respectively, following infection. The animals injected at 8 and 10 days showed cough and dyspnea following the injection. Those injected at 13, 17 and 19 days showed typical anaphylactic shock and died within 5 minutes of the injection.

Experiment 8.—Nine out of 12 guinea pigs were infected with 1000 larvae of *Trichinella spiralis*. One month later three were given 1 cc body fluid, three were given 0.05 g of extract A_1 and three were given the same amount of extract B_2. All injections were intravenous. All of them died of acute anaphylactic shock. The three uninfected guinea pigs were each given 1 cc of body fluid intravenously but showed no symptoms.

Experiment 9.—Eight guinea pigs were given 500 larvae of *T. spiralis;* at 20, 22, 24 and 27 days after infection one pair of animals was injected intravenously with 0.5 cc of body fluid. Those injected at 20 days showed no symptoms of shock, but those injected subsequently died of acute anaphylactic shock, except for one animal injected at 27 days, which recovered after showing marked symptoms.

Experiment 10.—Four guinea pigs were infected with 5000 eggs of *Parascaris equorum* and 13 days later were given 0.5 cc of the body fluid of *A. lumbricoides* intravenously. All died of acute anaphylactic shock.

Experiment 11.—Eight guinea pigs which were naturally infected with nematodes of the genus *Paraspidodera* were injected intravenously with 0.5 cc ascaris body fluid. All except one of these died of acute anaphylactic shock.

Experiment 12.—One group of guinea pigs was infected with 4000 eggs of *A. lumbricoides*, another group was infected with 500 larvae of *T. spiralis* and a third group was used as a control group. One month after infection 1% suspensions in 0.9% saline of the materials shown in table 3 were injected intravenously in 1 cc amounts into each animal. Table 3 shows the effect on infected animals caused by the injection. No effect oc-

curred in worm-free guinea pigs. One hour after the injection, if they had not already died, the guinea pigs were killed; and the animals were examined for emphysema of the lungs and for the presence of infection in the lungs or diaphragm. Infection was present in each animal infected previously. In all those animals which showed anaphylactic symptoms there was emphysema.

Experiments 7 to 12 show that guinea pigs infected not only with *A. lumbricoides* larvae but also with *P. equorum*, *T. spiralis* and nematodes of the genus *Paraspidodera* possibly become hypersensitive to the effects of ascaris body fluid. All the prepared ascaris extracts and tissues, the body fluid and also metabolic products become extremely toxic in ascaris-infected animals, causing typical anaphylactic shock in doses of 0.01 g, with sensitivity developing by 13 days after infection. A similar effect was produced by 0.01 g of *A. columnaris*, *Toxascaris leonina* and *Physaloptera maxillaris*. *T. spiralis* larvae caused dyspnea, but no symptoms were produced by *Ascaridia galli*, *Moniezia expansa*, *Macracanthorhyncus hirudinaceus*, oligochaetes or planorbid snails. No effect was produced by Forssman haptenes from guinea pig kidney, hemolyzed group A erythrocytes and pneumococcus polysaccharides, or from hyaluronidase or hyaluronic acid preparations. Trichina-infected guinea pigs became sensitive to ascaris body fluid at 22 days after infection. Their sensitivity to ascaris extracts A_1, A_2, and A_3, body fluid and metabolic products and to *P. maxillaris* was as acute as that in ascaris-infected guinea pigs.

TABLE 3.—*Effect of intravenous injection of various biological materials into guinea pigs infected with* A. lumbricoides *and* T. spiralis.

Material injected	Effect of intravenous injection		
	Ascaris-infected		Trichina-infected
Ascaris extract A_1	(4) +++		+++
Ascaris extract A_1 (boiled)	(2) +++	(2) ++	
Ascaris extract A_1 (digested)	(2) +++	(2) ++	
Ascaris extract A_2	(4) +++		+++
Ascaris extract A_3	(4) +++		+++
Ascaris extract A_6	(4) +++		++
Ascaris extract A_7	(2) +++		
Ascaris extract A_8	(2) +++		
Ascaris extract A_W	(2) +++		
Ascaris extract A_P	(2) +++	(2) +	
Ascaris extract A_{10}	(4) +++		
Ascaris ovary	(2) +++		+
Ascaris intestine	++		+
Ascaris muscle	++		
Ascaris cuticle	+++		
Ascaris body fluid	+++		+++
Ascaris metabolic products	+++		+++
Macracanthorhyncus hirudinaceus	−		
T. spiralis (larvae)	+		+
T. spiralis (infected diaphragm)	+		++
A. columnaris	+++		+
Toxascaris leonina	++		−
Ascaridia galli	−		−
Physaloptera maxillaris	+++		+++
Moniezia expansa	−		−
Lumbricus sp.	−		−
Tubifex sp.	−		−
Planorbis sp.	−		−
Pneumococcus polysaccharides*	−		−
Group A erythrocytes	−		−
Rabbit erythrocytes	−		−
Hyaluronidase (testicular extract)	−		−
Hyaluronic acid (vitreous humour)	−		−
Guinea pig lung (normal)	−		−
Guinea pig lung (infected with ascaris)	+++		++
Guinea pig kidney (normal)	−		−

Except for materials marked with an * (see methods), 0.01 g was injected in 1 cc physiological saline. One animal was injected with each preparation unless otherwise indicated in parentheses.
+++ signifies death from anaphylactic shock. ++ signifies anaphylactic symptoms followed by recovery. + signifies dyspnea. − signifies no symptoms.

Experiments on previously sensitized guinea pigs

Experiment 13.—Worm-free guinea pigs were subjected to sensitization with ascaris body fluid and later shocked with other materials. The mode of sensitization and subjection to shock are shown in table 4. In some cases the sensitizing dose consisted of a single injection; in others a second intraperitoneal injection was given. The second injection was made 6 to 7 days after the first in all such cases. In all cases the shocking dose was administered by intravenous injection.

Experiment 14.—Out of 10 guinea pigs which were sensitized by an intravenous injection of 2 cc of fresh body fluid, 2, 2, 1 and 1 of these were intravenously injected with 0.5 cc of ascaris body fluid at 7, 9, 11 and 13 days, respectively, after sensitization. Those animals injected at 7 days showed dyspnea, while those injected at 9, 11 and 13 days all showed definite anaphylactic shock. The animal injected at 13 days died within 5 minutes of the injection. Body fluid was administered in 4 different ways to the 4 remaining guinea pigs on the 13th day as follows: by inhalation, by intradermal injection, by instilling into the eye and by rubbing onto the shaved abdominal skin. No reaction was detectable in any animal, except coughing and dyspnea in the animal subjected to inhalation.

Experiment 15.—Twenty-five guinea pigs were sensitized by different routes of administration according to the protocol shown in table 5. Two percent lyophilized body fluid was used. The animals were exposed to 1% body fluid 3 weeks later, by 5 different routes of administration, and the effect is shown in table 6.

Experiment 16.—Fifteen guinea pigs

TABLE 4.—*Effect of intravenous injection of ascaris extracts (see table 1) in guinea pigs sensitized with ascaris body fluid.*

Number of animals	Sensitization material	Amount	Route*	Shocking material	Amount	Result
				i/v		
1	Fresh body fluid Fresh body fluid	(1) 0.3 cc (2) 0.5 cc	i.v. i.p.	Fresh body fluid	0.3 cc	+++
1	Fresh body fluid Fresh body fluid	(1) 0.3 cc (2) 0.5 cc	i.v. i.p.	Fresh body fluid	0.2 cc	+++
1	Fresh body fluid	(1) 0.3 cc (2) 0.5 cc	i.v. i.p.	Fresh body fluid	0.1 cc	+++
4	1% lyophilized body fluid	(1) 1 cc (2) 1 cc	i.p. i.p.	Body fluid Lyophilized	0.01 g	+++
5	1% lyophilized body fluid	(1) 1 cc (2) 1 cc	i.p. i.p.	A_1	0.01 g	+++
4	1% lyophilized body fluid	(1) 1 cc (2) 1 cc	i.p. i.p.	B_1	0.01 g	+++ (3), − (1)
2	1% lyophilized body fluid	(1) 1 cc (2) 1 cc	i.p. i.p.	A_1	0.01 g	+++ (1), + (1)
4	1% lyophilized body fluid	(1) 1 cc (2) 1 cc	i.p. i.p.	A_1	0.01 g	+++
3	1% lyophilized body fluid	(1) 1 cc (2) 1 cc	i.p. i.p.	A_p	0.01 g	−
3	1% lyophilized body fluid	(1) 1 cc (2) 1 cc	i.p. i.p.	A_s	0.01 g	+ (1), − (2)
3	1% lyophilized body fluid	0.8 cc	i.v.	Protein (body fluid)	0.01 g	+++
3	1% lyophilized body fluid	0.8 cc	i.v.	Crude polysaccharide (body fluid)	0.002 g	−
3	1% lyophilized body fluid	0.8 cc	i.v.	Filtrate (body fluid)	0.001 g	−

+++ signifies death from anaphylactic shock. + signifies slight dyspnea. − signifies no symptoms
* i.v.—intravenous. i.p.—intraperitoneal.

TABLE 5.—*Protocol for experiment 15.*

Number of animals	Route of sensitization	Days					
		1	2	3	4	5	6
5	Intravenous	0.5 cc		1.0 cc	1.0 cc	0.5 cc	
5	Intraperitoneal	0.5 cc	1.0 cc	1.0 cc		0.5 cc	
5	Subcutaneous	0.5 cc	0.5 cc	0.5 cc	0.5 cc	0.5 cc	0.5 cc
5	Oral	0.5 cc	0.5 cc	0.5 cc	0.5 cc	0.5 cc	0.5 cc
5	Inhalation	*	*	*	*	*	*

* Body fluid sprayed into enclosed space containing guinea pigs. Spraying continued for 5 minutes and guinea pigs left in atmosphere for 5 minutes after spraying.

were sensitized by 4 different routes of administration with 1% lyophilized body fluid by rubbing on the shaved abdomen, intraperitoneal injection (0.5 cc), oral dosage (0.5 cc) and by inhalation as in experiment 15. There were two sensitizing exposures, separated by a 4-day interval. Fourteen days after the last sensitizing exposure the animals were given 0.5 cc 1% lyophilized body fluid intravenously. Anaphylactic shock occurred only in the animals sensitized by intraperitoneal injection.

Experiments 14 to 16 showed that hypersensitivity to ascaris body fluid developed fully by 9 days following a single intravenous injection and that 0.1 cc of fresh body fluid was sufficient to cause death from anaphylactic shock. Animals were successfully sensitized to body fluid by intravenous, intraperitoneal and subcutaneous injection but not by rubbing the fluid on the skin, by inhalation or by oral administration. On the other hand, symptoms of hypersensitivity could only be elicited by intravenous injection. Experiment 13 showed that in animals sensitized to body fluid, death from anaphylaxis could be caused by intravenous injection of body fluid and whole worm extract A_1 and also by extracts from which protein had been removed by trichloracetic acid. No effect occurred after precipitation by phosphotungstic acid.

TABLE 6.—*Effect of body fluid secondarily administered by different routes to guinea pigs sensitized via different routes.*

	Method of secondary exposure				
	Intravenous	Intraperitoneal	Intradermal (subcutaneous)	Oral	Inhalation
Sensitization	0.5 cc	0.5 cc	0.1 cc	0.5 cc	Spray
Intravenous	+	−	−	−	−
Intraperitoneal	+	−	−	−	−
Subcutaneous (abdomen)	+	−	−	−	−
Oral	−	−	−	−	−
Inhalation	−	−	−	−	−

+ indicates death from anaphylactic shock.
− indicates no symptoms.

DISCUSSION

The experiments described above have demonstrated, firstly, that a substance causing anaphylactoid symptoms in worm-free guinea pigs is associated with the tissues of *A. lumbricoides* and, secondly, that in animals sensitized with ascaris body fluid and also in those infected with ascaris or trichina larvae, protein-free ascaris materials will cause anaphylactic shock. The former substance appears to be associated with the proteins of the worm because it is mostly removed by protein precipitants, by boiling and by complete digestion with pepsin. Its effect is only observed following the intravenous injection of relatively very large doses, and, unlike anaphylaxis, the severity of the symptoms and rapidity of death appear to depend primarily on the amount of material injected. The most prominent symptom is dyspnea, which apparently results from constriction of the bronchioles, because marked emphysema is observed at autopsy. In the small number of animals injected with different tissues of ascaris it appeared that the location of this substance is the

cuticle, and to a lesser extent the muscle and ovary; it was not present in the body fluid or intestine. Previous observations on the toxicity of ascaris tissues and fluids have already been discussed (Sprent, 1949b), and it was suggested that many of the instances where toxic effects resembling anaphylactic shock were observed following intravenous administration of ascaris materials were really manifestations of hypersensitivity resulting from infection or some other antigenic stimulation. In spite of the observations made here that ascaris tissues contain a toxic substance active in nonsensitized animals, this hypothesis is still upheld. It appears likely that the toxic effect observed with whole worm pulp is a form of nonspecific protein shock, an effect which is possibly common to many invertebrate proteins when injected in large amounts into the bloodstream of mammals. There seems to be no support to the hypothesis that the effect is caused by a specific ascaris toxin because such large amounts (approximately 1 g/kg body weight) are necessary to produce the effect. Furthermore, the observations that relatively very small amounts of ascaris extracts, even after removal of protein, will cause typical anaphylactic shock in guinea pigs infected with ascaris or trichina larvae tend considerably to support the contention that when ascaris materials have been observed to cause shock in animals such as dogs and horses, most of which at some time have been infected with ascarid worms, the observed effects have been allergic in character. The effects observed in guinea pigs following intravenous injection of deproteinized ascaris extracts by Macheboeuf and Mandoul (1939), Rocha e Silva and Grana (1946) and Deschiens and Poirier (1947) were not observed by the writer in worm-free guinea pigs. It is possible that the guinea pigs used by the above workers were infected with nematodes, although they do not mention whether any search for parasites was made. The observation by the writer that a natural parasite of the guinea pig, a nematode of the genus *Paraspidodera*, appeared to render the animals sensitive to ascaris body fluid may be significant in this respect, as also may be the observation by Deschiens and Poirier that antihistaminic drugs were effective in preventing the symptoms of shock. The accidental sensitization of animals to ascaris materials cannot be ignored in this connection. This might result from failure to sterilize cages occupied by animals experimentally infected with ascaris eggs. These eggs are passed in large numbers in the feces of guinea pigs and mice following oral administration and are capable of infecting other animals which eat the feces with their food (unpublished results).

Regarding the chemical nature of the substance or substances causing shock in infected animals, no definite statement is possible until chemically pure materials are obtained. It can, however, be concluded that although protein is probably one of the substances involved, it is not the only one, because deproteinized ascaris extracts are also effective. It will be observed from table 1 that all the extracts used in these studies contained some nitrogenous material with the exception of the filtrate after phosphotungstic acid precipitation (A_p). This extract (A_p) probably contained mostly polysaccharides; it was readily soluble in water to form an opalescent solution. However, as one preparation of A_p was found to contain a small amount of nitrogen, its activity cannot as yet be considered to reside in its non-nitrogenous constituents. Furthermore, some preparations of this extract caused very little effect

in infected guinea pigs. The other extracts evidently all contained nitrogenous material but probably also contained polysaccharides. An attempt was made to remove the polysaccharides from extract A_2 by alcohol. Although much of it was precipitated as extract A_x, the filtrate (extract A_3) was not perfectly clear even after several reprecipitations with alcohol, and some polysaccharide was probably present. The same may be said for the extract E_1 of Rocha e Silva and Grana (1946) and the similar extract B_4 used in the present studies, which was obtained by precipitation with acetone after the worm pulp had been treated with trichloracetic acid and alcohol. These extracts were probably contaminated by polysaccharide material because acetone would be expected to precipitate any polysaccharide not removed by the alcohol. As saturation with ammonium sulphate precipitates glycogen, it is likely that extracts A_w and A_{2w} contained polysaccharides also. Extract A_x was probably not pure polysaccharide because it contained nitrogenous constituents. Campbell (1936) found that they could be removed from the ascaris polysaccharide by phosphotungstic acid precipitation. The nitrogenous substances present in the filtrate after trichloracetic acid precipitation were possibly proteose and peptone, as these substances are not, according to Wasteneys and Borsook (1924), precipitated by trichloracetic acid. The slight dyspnea following injection of extracts A_2 and A_3 in nonsensitized animals may have been caused by these substances because after partial digestion with pepsin (see table 2), this effect was more pronounced following injection of extracts D_2 and D_3. The observation that ascaris body fluid and worm pulp after being kept at room temperature for 18 hours caused a greater degree of dyspnea than the same substances kept in the refrigerator may have been caused by an increase in the proteose and peptone content as a result of proteolysis which may have resulted either from bacterial activity or from the activity of proteolytic enzymes in the worm. Such enzymes have been described by Sang (1938). Whether proteoses and peptones may be considered as contributing to the toxicity of ascaris extracts from fresh material in nonsensitized animals is doubtful because of the very slight toxicity of extracts after trichloracetic acid precipitation. Such products of protein hydrolysis have been incriminated as the cause of toxicity in ascaris extracts by Shimamura and Fujii (1917) and Rocha e Silva et al (1946), but in view of the doubt as to whether they were observing allergic or primarily toxic effects these observations cannot be considered as evidence for their toxicity in nonsensitized animals. Whether they are involved in the production of anaphylactic shock in sensitized or infected animals is also open to doubt because of the difficulty of extracting them free of polysaccharides.

It can be said, therefore, that of the three different substances under consideration, i.e., proteins, polysaccharides and hydrolytic breakdown products of protein, the effect of extract A_y supports the involvement of the first of them in the cause of anaphylactic shock in sensitized or infected guinea pigs. The implication of the second and third awaits the isolation of purer extracts. It is likely that if animals are sensitized through infection the sensitizing antigen must be released from the invading larvae to stimulate the production of sensitizing antibodies. It seems likely, therefore, that whatever substance is involved is present in the excreted metabolic products of the worms, and the shock produced in infected animals

by adult metabolic products confirms this hypothesis. The fact that such products caused shock both in an ascaris- and a trichina-infected guinea pig suggests that the antigen concerned may be widely distributed among the helminths, and the activity of extracts made from several other nematodes lends further support. It is interesting in this connection to consider the observation of Oliver-González (1946) that an increase of α-agglutinins occurred in the serum of ascaris-infected rabbits. This phenomenon may have been due to the ascaris polysaccharide because this polysaccharide, according to Oliver-González, will absorb α-agglutinins from human serum. It appears from his work that an immunological relationship exists between the polysaccharides from several different helminths, the group A specific substance in human blood, the Forssman haptene and the pneumococcus polysaccharides. If one of a widely distributed series of immunologically related polysaccharides is released during infection by ascaris larvae, it might well be expected that any preparation containing such materials would cause anaphylactic shock when injected into infected animals, as Campbell (1936) has already shown that the purified ascaris polysaccharide is capable of sensitizing guinea pigs. In the present series of experiments no shock was produced in infected animals by pneumococcus polysaccharides, hemolyzed group A erythrocytes or by guinea pig kidney. Nevertheless, further experiments with these materials in guinea pigs sensitized with pure ascaris polysaccharide are in progress.

From these studies it would appear that the mechanism involved in hypersensitivity of infection and in experimental hypersensitivity to certain antigenic materials is identical, the sensitivity of the infected animal depending on the nature of the antigens excreted by the parasite. As it appears likely that many helminths excrete immunologically related substances, it follows that the diagnosis of specific helminthiasis by allergic tests is subject to considerable error. Cross sensitization occurring with ascaris and trichina infections in man has been observed previously by Baron and Brunner (1942), and both Maternowska (1934) and Schonfeld (1937) have concluded that sensitivity to ascaris materials may follow infection with nematodes of the orders Ascaroidea, Trichuroidea and Oxyuroidea.

In a previous publication (Sprent and Chen, 1949a) the importance of precise knowledge concerning metabolic products of helminths was pointed out, insofar as such knowledge may lead to the discovery of the antigens which instigate immunity. Similarly, such knowledge may lead to the discovery of species-specific antigens for use in diagnosis.

SUMMARY

Guinea pigs were injected intravenously with various ascaris tissues and fluids and also with different chemical extracts prepared from whole worm pulp. In nonsensitized worm-free guinea pigs a toxic effect resembling anaphylactic shock was observed following injection of the supernatant fluid from centrifuged worm pulp in doses of about 1 g/kg body weight. Removal of proteins by protein precipitants, boiling and complete digestion by pepsin removed this toxic activity, suggesting that the toxic substance is a protein. It was not removed by dialysis. Some dyspnea was caused by the deproteinized extracts, and this latter effect may have been caused by products of protein hydrolysis as it was more marked after partial digestion with pepsin. When ascaris body fluid and whole

worm pulp were left at room temperature for 18 hours, an increase in toxicity occurred which may have been due to such substances released by proteolytic activity.

Guinea pigs could be sensitized with ascaris body fluid by intravenous, intraperitoneal and subcutaneous injection, while inhalation of a mist of body fluid, oral administration and rubbing onto the abdominal skin failed to produce sensitization. Allergic reactions could be obtained only by intravenous injection and in one case by inhalation. Oral, subcutaneous and intraperitoneal administration failed to produce any effect. Guinea pigs sensitized to ascaris body fluid were successfully shocked with ascaris extracts deproteinized by trichloracetic acid but not after phosphotungstic acid precipitation.

Guinea pigs infected with the larvae of *Ascaris lumbricoides*, *Parascaris equorum* and *Trichinella spiralis* and with nematodes of the genus *Paraspidodera* were found to be hypersensitive to ascaris body fluid. Whole worm and deproteinized extracts of *A. lumbricoides*, isolated ascaris tissues and metabolic products; whole worm preparations of adult *A. columnaris*, *Toxascaris leonina* and *Physaloptera maxillaris;* and guinea pig lung containing ascaris larvae all caused anaphylactic shock in guinea pigs infected with *A. lumbricoides*. Pneumococcus polysaccharides, hemolyzed group A erythrocytes, normal guinea pig kidney and lung, hyaluronidase and hyaluronic acid preparations, and whole preparations of *Macracanthorhyncus hirudinaceus*, *Moniezia expansa*, oligochaete worms and a planorbid snail failed to produce shock. Guinea pigs infected with *T. spiralis* were successfully shocked with whole worm and deproteinized ascaris extracts.

Regarding the chemical nature of the substance causing shock in infected animals, it appears possible that both protein and polysaccharide are involved. The occurrence of widely distributed immunologically related polysaccharides is discussed in connection with their possible role in instigating cross sensitization in infections with various helminths. The importance of finding a means of analyzing the metabolic products of helminth parasites is stressed, insofar as such studies may assist in discovering more specific antigens for use in allergic methods of diagnosis.

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ANTIGENICITY OF CELL-FREE STAPHYLOCOCCAL COAGULASE

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The fact that certain strains of staphylococci have the ability to clot plasma was recognized in 1903,[1] and since then this reaction has been widely employed as a test for pathogenicity of the organism. From the investigations of Smith and Hale[2] and Kaplan and Spink[3] the mechanism whereby staphylococcal coagulase clots plasma has been elucidated to some extent. Most strains of *Staphylococcus aureus* produce an extracellular substance, coagulase, during their growth. Coagulase does not clot fibrinogen directly; a second factor, normally found in the blood of many species, is required in the reaction. This factor was termed "activator" by Smith and Hale[2] and "coagluase reacting factor" by Tager.[4] Since staphylococcal coagulase has not been identified as the active clotting substance, the term activator is somewhat misleading. It would appear equally possible that the factor found in blood is acted on by staphylococcal coagulase to produce a clotting enzyme.

Considerable interest has been exhibited in coagulase from the standpoint of its role in infections caused by *Staph. aureus*. Kemkes[5] thought that staphylococcal coagulase might be antigenic but failed to demonstrate an inhibitor in the blood of 5 subjects with staphylococcal infections, or in antistaphylococcal serum. Gross[6] failed to detect anticoagulase in rabbits injected with viable staphylococci. Sudhues and Schimrigk[7] observed no difference in the time required to clot the plasmas of normal individuals and plasmas from persons with staphylococcal infections. Walston[8] injected two rabbits with cell-free coagulase and failed to produce plasma resistant to clotting. Cruickshank[9] studied 4 patients with chronic staphylococcal infections of the skin who had been given a course of staphylococcal toxoid. They developed an increase in the antihemolysin titer of the blood, but their plasma failed to show increased resistance to clotting with staphylococcal coagulase. Likewise, the plasma of a patient with osteomyelitis and a patient with staphylococcal pneumonia and empyema showed normal clotting times when tested with coagu-

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We wish to express our appreciation to Miss Margaret M. Hezebicks for her technical assistance.

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6. Gross, H. 1931, Zentralbl. f. Bakt. **122**: 354–361.
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lase. Smith and Hale[9] were unable to immunize rabbits or guinea pigs with coagulase.

In contrast with these negative results are those reported by Gross,[10] who found that some human serums and antistaphylococcal rabbit serums were capable of inhibiting the clotting action of coagulase on plasma. Here, however, such inhibition was exhibited only in low dilutions of the serums. He also noticed, as have others[2,11,12] that the plasma of some humans failed to clot upon the addition of coagulase. These results suggested that staphylococcal coagulase might be antigenic.

More recently Tager and Hales[13] injected 6 rabbits with a highly purified and concentrated cell-free coagulase preparation and demonstrated an increased resistance to clotting of the plasma in 5 of the animals. A preliminary report from this laboratory[14] showed that an increase in titer of anticoagulase was demonstrable in monkeys injected with cell-free coagulase.

The present study reports the production of an antibody to cell-free coagulase in animals.

METHODS

Coagulase.—Cell-free coagulase was prepared from a number of coagulase-positive strains of *Staph. aureus* isolated from patients with infections and from pharyngeal cultures of normal subjects. The exact method of preparation of the coagulase varied, but in general 500 ml of tryptose broth containing either 5% human plasma or 5% bovine albumin were inoculated with 5 to 10 ml of an overnight broth culture of the test strain. After incubation at 37 C for three days the culture was centrifuged to sediment the cells and the supernatant fluid filtered through a Seitz pad. The first 50 ml to pass through the filter were discarded. The filtrate was tested for sterility and stored in sealed glass ampules at −20 C or 4C until use.

The preparations of coagulase were titrated by making serial two-fold dilutions in tryptose broth in a volume of 1 ml. Following this, 1 ml of a bovine fibrinogen* solution, containing 100 mg per 64 ml of phosphate buffer at pH of 7.4, was added to each tube containing the plasma preparation of coagulase. The solution was mixed and incubated at 37 C for three hours in a water bath. The highest initial dilution which clotted the fibrinogen was taken as an indication of the degree of activity of the preparation. Coagulase prepared in plasma contains an unknown amount of reacting factor, and since clotting is dependent upon the presence of coagulase and the reacting factor, the activity of coagulase in such a preparation cannot be measured accurately.

The amount of coagulase present in broth cultures containing bovine albumin instead of plasma may be accurately determined, since the albumin used contained no reacting factor.[15] After making the proper dilutions of the preparation to be tested, as given above, 1 ml of a standard fibrinogen solution containing a constant amount of reacting-factor is added. This fibrinogen solution is prepared by adding 100 mg of bovine fibrinogen to 63 ml of phosphate buffer. Following incubation at 37 C for 30 minutes, 1 ml of a solution containing 0.1 mg of heparin is added. The coagulase reacting factor is then added in the form of a standard human serum in a final dilution in the fibrinogen solution of 1:3500. After the addition of the fibrinogen to the coagulase preparation the solutions are mixed and incubated in the water bath for three hours at 37 C. The tubes are then immediately examined with a hand lens for evidence of clot formation. A final titration is performed beginning with the highest dilution of coagulase which produces a visible clot. In this procedure decrements of 0.1 ml are employed, the volume being brought up to 1 ml with tryptose broth. A unit of coagulase is defined as the smallest amount of coagulase in a volume of 2 ml which will clot the standard fibrinogen-reacting factor preparation after three hours incubation at 37 C.

Immunization procedures.—Rabbits, chickens,

10. Gross, H. 1933, Klin. Wchnschr. **12**: 304–306.
11. Lominski, I. and Roberts, G. B. S. 1946, J. Path. & Bact. **58**: 187–197.
12. Tager, M. and Hales, H. B. 1948, Yale J. Biol. & Med. **21**: 91–97.
13. Tager, M. and Hales, H. B. 1948, J. Immunol. **60**: 475–485.
14. Rammelkamp, C. H. 1948, Am. J. Med. **4**: 782.

* Armour and Company, Chicago, Ill.

15. Rammelkamp, C. H., Jr., Badger, G. F., Dingle, J. H., Feller, A. E., and Hodges, R. G. 1949, Proc. Soc. Exper. Biol. & Med. **72**: 210.

ducks, geese, and *Macacus rhesus* monkeys were used as experimental animals. All injections of cell-free coagulase were administered intramuscularly. Usually the animals received a series of injections for 3 periods of 5 or 6 days each, with a rest of one week between each series. Blood was obtained before immunization and approximately one week after each series of injections. After clotting, the serum was removed and stored in sterile vials at 4 C until tested.

Measurement of anticoagulase.—A modification of the Lominski and Roberts[11] method for the determination of the titer of the coagulase-inhibitor in the serum has been described in detail elsewhere.[16] Before testing, the serums were inactivated at 60 C for 20 minutes, following which they were placed at 4 C for 60 minutes and then inactivated a second time for 20 minutes at 60 C. All serums from one animal were tested at the same time.

RESULTS

Rabbits.—Six rabbits received cell-free coagulase. Each animal was injected intramuscularly with 18 ml of coagulase divided into three weekly courses, with a week of rest between each series. The total number of units used in each animal was 900. In no instance was there a rise in the antibody titer of the serum specimens obtained during and following the injections.

Chickens.—Nine chickens were injected with coagulase prepared in tryptose broth containing plasma. These coagulase preparations clotted the standard fibrinogen solution in a dilution of 1:25 to 1:57. The amount injected varied from 2.5 to 36 ml. Only one of the 9 chickens showed an increase in coagulase-neutralizing capacity of the serum. The titer before inoculation was 7.5 and 59 days later was 25. These serums were tested in 1947 and repeated in 1949 with similar results.

A series of serums collected from each of 6 chickens who received no injections of coagulase showed no increase in anticoagulase.

Ducks and geese.—Two ducks were given a total of 13 ml of coagulase divided into 6 injections. This coagulase was prepared from a plasma-tryptose broth culture and clotted the standard fibrinogen in a dilution of 1:44. Neither duck developed anticoagulase in the serums obtained following this course of injections.

A year later a second attempt was made to immunize birds. For this purpose cell-free coagulase prepared from tryptose broth containing albumin was used. A total of 120 ml was administered intramuscularly in 12 injections to 2 ducks and 2 geese. The total amount of coagulase given to each animal was 6804 units. No antibody could be demonstrated in the serums of these animals.

TABLE 1.—*Anticoagulase titers of monkey serums following immunization with coagulase prepared in plasma broth.*

Date	Strength of coagulase	Total injected (ml)	Antibody titers		
			Monkey no.		
			1	2	3
6/18/47			11	25	125
7/14–7/19	1:27.3	12			
7/25			7.5	900	625
7/28–8/2	1:27.3	12			
8/9			<5	1400	900
8/11–8/16	1:27.3	12			
8/25			17	900	
9/2			11	417	417
			Monkey no.		
			4		5
4/22/47			7.5		<5
4/23–5/12	1:19	19			
5/16			1400		<5
5/26			625		<5
8/2			125		<5
9/2			56		<5

Monkeys.—In the first two attempts to immunize monkeys, cell-free coagulase prepared from tryptose broth containing plasma was used. The results are presented in table 1. Following the injections of coagulase the serums of 3 of the 5 monkeys exhibited an anticoagulase effect.

These results obtained with a coagulase preparation containing the reacting factor could be explained by the development of an antibody either to coagulase or to reacting-factor. They could not be explained by quantitative

variations in the reacting-factor in the monkey serums tested since this substance was destroyed by heat (inactivation). Although it appeared unlikely that an antibody to the reacting-factor was responsible for the results shown in table 1, further experiments were designed to investigate the point. Evidence was obtained in two ways: by using cell-free coagulase containing no reacting-factor for the inoculation of monkeys, and by attempting to develop antibodies to the reacting-factor in a monkey.

TABLE 2.—*Immune response of monkeys to intramuscular injections of coagulase free of reacting-factor.*

Monkey no.	Cell-free coagulase injections		Anticoagulase titer
	Day	Units	
B	0	58	<25
	1	58	
	3	58	
	10		625
	14	58	
	17	58	
	20		900
C	0		36
	1	214	
	2	214	
	3	214	
	4	214	
	5	214	
	13	214	
	14	214	
	15	214	
	16	214	
	17	214	
	24		1400
	30		900
	40		625
G	0		<25
	1	125	
	3	125	
	10		278
	14	125	
	17	125	
	22		900

Cell-free coagulase containing no reacting-factor was used as the antigen to immunize three monkeys. The organisms were grown in broth containing albumin, and the serums from each animal were tested with the homologous coagulase preparation. Animal B was injected with coagulase prepared from a strain (Lindsay) of *Staph. aureus* recently isolated from a boil and monkey C with coagulase prepared from strain 104 obtained from Tager.[16] The third monkey (G) was immunized with coagulase obtained from a strain isolated from the oropharynx. The results are shown in table 2. Each animal exhibited an increase in the coagulase-neutralizing capacity of the serums collected during and following the immunization procedure. Since the reacting-factor was not used in the immunization of the animals, only anticoagulase could be held responsible for the results obtained.

One monkey was inoculated with the coagulase reacting-factor in the form of human serum (Henderson) in amounts of 10 ml per injection. Three injections were given intramuscularly during the first week, and, following a rest period of one week, a second series of three injections was given. Two preinoculation and three postinoculation serums were tested for evidence of an antireacting factor.

The serums were inactivated twice at 60 C, and phosphate buffer was added to make a final dilution of 1:10. Twofold serial dilutions of each serum were then made in volumes of 0.5 ml. To each tube was added 0.5 ml of a 1:1000 dilution in phosphate buffer of the serum reacting-factor (Henderson). Following incubation at 37 C for 60 minutes, 0.5 ml of fibrinogen solution was added. This solution contained 100 mg bovine fibrinogen and 0.1 mg of heparin in 32 ml of phosphate buffer. One unit of cell-free coagulase in 0.5 ml of broth was added to each tube. The tubes were then incubated at 37 C for three hours and examined for clot formation. No evidence was obtained for the development of an antibody to the reacting factor in the serum of this monkey.

DISCUSSION

Rabbits were used as the test animal

16. Tager, M. 1948, Yale J. Biol. & Med. **20**: 487–501.

in the initial attempts to demonstrate an antibody to coagulase. Of 6 rabbits which received cell-free coagulase, none developed antibodies to coagulase. These results are not surprising in view of the fact that other investigators have failed to demonstrate antibodies to coagulase in these animals.[2,6,8] The only successful experiment employing rabbits is that reported by Tager and Hales,[13] and in spite of the use of relatively large amounts of cell-free coagulase, no antibodies were demonstrated in the serums of three rabbits during the initial immunization period. With the use of an adjuvant, however, three rabbits developed antibodies to coagulase during the initial immunization period. These results indicate that rabbits respond poorly to injections with coagulase.

Attempts to immunize guinea pigs have failed to demonstrate an anticoagulase.[2] Likewise, in the experiments reported here, ducks, geese, and chickens failed to develop a high titer of antibodies to coagulase after injection of cell-free coagulase. One chicken showed a slight increase in the anticoagulase titer of the serum after immunization.

The response of monkeys to the injection of cell-free coagulase is in striking contrast to that obtained in rabbits, chickens, ducks, and geese. During the course of this and other investigations, a total of 17 monkeys has been injected with such preparations, and 11 have developed anticoagulase during the initial period of immunization. In all of these experiments relatively small amounts of coagulase were injected, and yet the change in the anticoagulase titer of the serum was definite. It is entirely possible that by the use of more potent immunizing preparations all animals will respond.

That the inhibitory effect of the serum specimens collected following immunization with coagulase is due to a specific antibody appears to be well established, although Smith and Hale[2] have suggested that variations in the susceptibility of various plasmas to clotting by staphylocoagulase are due to changes in the activator content of such plasmas and not to the presence of an anticoagulase. Tager and Hales[13] in their experiments could not demonstrate a change in the reacting factor of the blood of rabbits during immunization and yet were able to show complement-fixing antibodies, collodion-particle agglutination, and coagulase-neutralizing antibodies in the serum and plasma. In the studies presented in this report an attempt was made to immunize a monkey with large amounts of human reacting-factor, but no antibodies were demonstrated. The anticoagulase effect of the serums of immunized animals was not due to quantitative changes in the reacting-factor, since the serums to be tested were first heated to remove the reacting factor and then added to a system containing a constant quantity of human reacting factor.

SUMMARY

Rabbits, chickens, ducks, geese, and monkeys were inoculated with cell-free coagulase. No antibodies were demonstrated in the serum of the rabbits, ducks, and geese. The serum of one chicken showed a slight increase in anticoagulase titer following immunization.

Eleven of 17 monkeys developed coagulase-neutralizing properties in their serums following immunization with cell-free coagulase. The available evidence indicates that this inhibitory substance, which may be termed "anticoagulase," is a specific antibody to coagulase.

THE SPREAD OF GROUP A STREPTOCOCCI IN THE BARRACKS OF NAVAL PERSONNEL

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The relative importance of the carrier of Lancefield group A streptococci in the spread of acute streptococcal infection remains incompletely defined. In military personnel, Dudley[1] incriminated the sleeping quarters as an important focus in the spread of these infections. Hamburger et al,[2–5] from observations on streptococcal disease in army personnel, have presented evidence directing strong suspicion toward the individual who harbors streptococci in both the nose and throat as the "dangerous carrier." These studies have shown that such an individual is more likely to be a "streptococcal disperser" than one who harbors the organism only in the throat and that such "dispersers" are capable of heavily contaminating their hands, clothing, bedding and floor dust in their immediate enviroment. Further, they observed that focal outbreaks of streptococcal respiratory infections in the army units under observation occurred in a pattern suggesting that the infections were acquired in the sleeping quarters rather than through personal contact or by their intermingling at other locations. The presence of large numbers of group A streptococci in the floor dust, bedding and air of the barracks occupied by units suffering outbreaks of streptococcal infections of the same type as recovered from the throats of barrack inhabitants, suggested that the inhalation of dust-borne organisms was responsible for such outbreaks.

At this station observations were made during the winter of 1947–48 upon respiratory diseases which occurred in a large population of naval recruits.[6] The focal nature of outbreaks of infections due to group A streptococci had been apparent in previous winters, and op-

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The technical assistance of J. T. Prince, T. L. Sidener, J. E. Gretzinger and W. P. Hebert, Hospital Corps, U. S. Navy, is gratefully acknowledged.

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2. Hamburger, M., Jr., Puck, T. T., Hamburger, V. G. and Johnson, M. A. 1944, Studies on the transmission of hemolytic streptococcus infections. III. Hemolytic streptococci in the air, dust and bedclothing of hospital wards and their relation to cross infections. J. Infect. Dis. **75**: 79–84.
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4. Loosli, C. G., Lemon, H. M., Wise, H. and Robertson, O. H. 1948, Studies on the transmission and control of respiratory disease within army barracks: I. Hemolytic streptococcal reservoirs and their relation to the nose and throat flora of military personnel. J. Infect. Dis. **82**: 59–71.
5. Lemon, H. M., Loosli, C. G. and Hamburger, M., Jr. 1948, The transmission and control of respiratory diseases in army barracks. II. The spread of hemolytic streptococcal infections among enlisted personnel. J. Infect. Dis. **82**: 72–85.
6. Jarrett, E. T., Langmuir, A. D. and Hollaender, A. 1948. Studies of the control of acute respiratory diseases among naval recruits: V. Ultraviolet irradiation in control of airborne contamination in naval barracks. To be published.

portunity was sought for study of the patterns of spread within the barracks occupied by companies in which outbreaks occurred. For this purpose, the first two companies presenting evidence of an outbreak of streptococcal infection during the winter were selected. Both companies experienced severe outbreaks of acute respiratory infections during which the recovery of group A type 10/12 streptococci from the nose of both admitted cases and carriers was a prominent feature. Five other types of streptococci were prevalent in carriers of one of these two companies but were of negligible importance in those who became clinically ill. These types were apparently of low virulence in comparison with type 10/12, as evidenced by their behavior in these companies and subsequently in the regiment as a whole.

MATERIAL AND METHODS

This special study was done as a part of research project NM 005 008, "The Evaluation of Ultraviolet Radiation in the Control of Communicable Disease in Naval Barracks," at the U. S. Naval Training Center, Great Lakes. The general description of the population, the environmental background and the over-all results are presented elsewhere.[6] All cases of illness presenting themselves for treatment were seen by one medical officer, who used the criteria of the Commission on Acute Respiratory Disease[7] in assigning diagnoses, and all with a fever of 100 F or over were admitted. Routine nose and throat cultures were obtained in all admissions and the swabs streaked directly on petri plates of proteose peptone no. 3 agar, pH 7.5, containing 5% human blood with gentian violet, one part per million. Air sampling technics are described elsewhere.

Throughout this report, the terms "streptococci" or "type —" refer only to Lancefield group A streptococci. All grouping and typings were done by the same individuals, using commercially prepared serums following the Lancefield technique.[8] The term "carrier" as used throughout implies only that the individual harboring streptococci in the nose or throat was serving in a full-duty status at the time of survey.

7. Commission on Acute Respiratory Diseases. 1944, Endemic exudative pharyngitis and tonsillitis. Etiology and clinical characteristics. J.A.M.A. **125**: 1163–1169.

RESULTS

The experience of the two companies selected for study was as follows:

Company A.—This company was organized on January 3, 1948, and there were no admissions for illness during the first week. On January 10th, the 117 men entered the regiment, and an outbreak of acute respiratory infections began on the following day. Thirty-one (26% of the 117 men) were admitted to the dispensary within the next three weeks. Twenty-two were diagnosed as acute undifferentiated respiratory disease (ARD), four others as primary atypical pneumonia (PAP). Nose and throat cultures were obtained on all admissions, and a few type 10/12 streptococci were recovered from the throats of six men, in only one of whom was there

TABLE 1.—*Summary of dispensary admissions, streptococcus carriers and distribution of types of group A streptococci recovered from Company A.*

	Admitted Cases				
	ARD	PAP	EXP	Total	Carriers
(a) First phase, January 12–30, 1948					
Number admitted	22	4	5	31	
Number cultured	22	4	5	31	109
Number of positive cultures	5	1	1	7	5
Number of type 10/12					
Nose	0	0	0	0	2
Throat	4	1	1	6	2
Number of type 18					
Nose	0	0	0	0	0
Throat	1	0	0	1	2
Number of type 19					
Nose	0	0	0	0	0
Throat	0	0	0	0	1
(b) Second phase, February 8–25, 1948					
Number admitted	9	0	10	19	
Number cultured	9	0	10	19	100
Number of positive cultures	4		7	11	28*
Number of type 10/12					
Nose	3		5	8	8
Throat	4		7	11	28

ARD indicates a diagnosis of acute undifferentiated respiratory disease; PAP, primary atypical pneumonia; EXP, exudative tonsillitis or pharyngitis.

* Includes 4 men positive and 3 men negative upon admission during second phase outbreak.

8. Swift, H. F., Wilson, A. T. and Lancefield, R. C. 1943, Typing group A streptococci by M precipitin reaction in capillary pipettes. J. Exper. Med. **78**: 127–133.

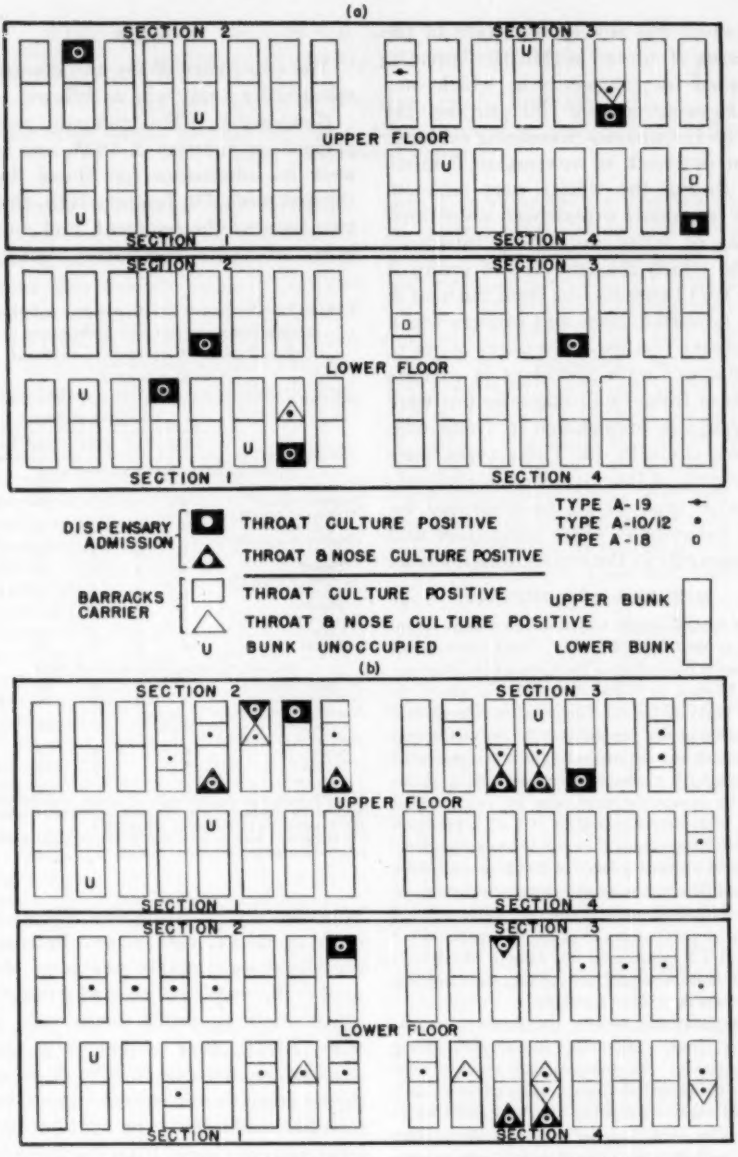

FIG. 1—Spread of type A 10/12 hemolytic streptococci in barracks of Company A.

clinical evidence of a streptococcal infection. Upon the subsidence of the outbreak, there were no further admissions from the company for another 8 days.

A survey for carriers of streptococci was made on January 21st at the height

of the above outbreak. Types 18 or 19 were recovered from the throats of three men and type 10/12 from both the nose and throat of two others. The distribution of streptococcal types from dispensary-admitted cases and barrack carriers is shown in table 1 (a). The dormitory sleeping billets occupied by admitted cases and carriers yielding positive cultures during this phase of the company experience are shown in figure 1 (a).

On February 8th, 8 days following the outbreak described above, a second outbreak of acute respiratory infections began with 19 new admissions during the ensuing 17 days. Nearly pure cultures of type 10/12 streptococci were obtained from the throats of 11 (58%) of these admitted cases, 8 of whom also presented the same type in their nasal cultures. Type 10/12 streptococci were also recovered from 4 of 10 samples of air collected in this company's barracks on February 18th.

A second carrier survey was conducted on February 25th, during the company's 8th week in training and following the subsidence of this last outbreak. Twenty-eight carriers of streptococci were found among the 100 men who remained. Both nose and throat cultures were positive in 8 men, in 4 of whom the same type of streptococcus had been recovered upon their dispensary admission two weeks previously. All strains of streptococci isolated during this second outbreak and survey in the company were type 10/12, and this type was responsible for all positive nasal cultures in this company's experience.

A summary of the results during this second episode is presented in table 1 (b), and the distribution of dormitory bunks is shown in figure 1 (b). Over 40% of the men in the company were admitted during their first 8 weeks of training because of acute respiratory disease.

Company B.—The experience of this recruit company composed of 115 men who began training on January 5th, two days after Company A, was distinctly different. In the three-week period between their arrival and January 25th only three men were admitted to the dispensary with acute respiratory disease. A type 24 streptococcus was isolated from the throat culture of one of these men. Beginning January 25th and continuing for 6 weeks until March 5th, an outbreak of respiratory disease occurred during which 36% of the company was admitted to the dispensary. Twenty-two of these were diagnosed as acute undifferentiated respiratory disease (ARD) and 20 were diagnosed as exudative tonsillitis-pharyngitis (EXP). As is depicted in table 2, streptococci were isolated from the throat only in 3 of the 22 cases of acute undifferentiated respiratory disease. Numerous streptococci were isolated from the throats of 12 of the 20 cases of exudative tonsillitis-pharyngitis, and the same type of

TABLE 2.—*Summary of dispensary admission, streptococcus carriers and distribution of specific types of streptococci in Company B.*

	Admitted Cases			
	ARD	EXP	Total	Carriers
Number admitted	22	20	42	
Number cultured	22	20	42	102
Number of positive cultures	3	42	15	30*
Number of type 1				
Nose	0	0	0	1
Throat	1	0	1	7
Number of type 3				
Nose	0	0	0	2
Throat	1	1	2	13
Number of type 10/12				
Nose	0	5	5	2
Throat	1	9	10	5
Number of type 18				
Nose	0	1	1	0
Throat	0	2	2	1
Number of type ?				
Nose	0	0	0	0
Throat	0	0	0	4

* Includes 9 men who had recent respiratory illness; 4 were positive and 5 negative for streptococci on admission cultures.

streptococcus was isolated from the nose of 6 of the 12 men.

A carrier survey was conducted on March 5th at the termination of the outbreak. Streptococci were recovered from the throats of 30 men (29.4%), and several foci which accounted for most of the organisms recovered. Six of the 13 cultures for type 3 and one of the two admitted cases were billeted in the distal end of sections 1 and 2 among the bunks of 14 men. Five of the 8 positive

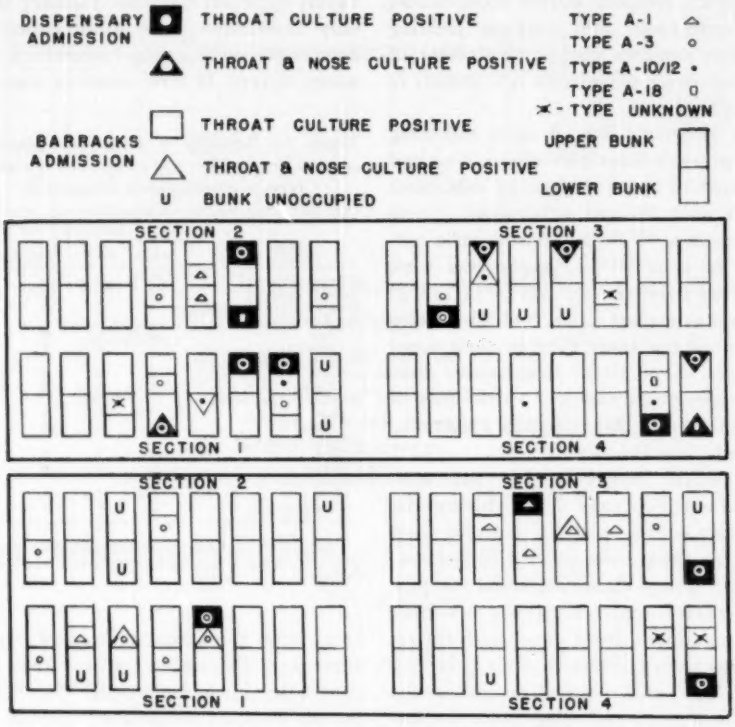

FIG. 2.—Spread of multiple types of group A streptococci in barracks of Company B.

the same type was recovered from the nose of 5 of these. The same type of streptococcus was recovered at the time of an earlier admission and at survey in 5 men. Air sampling recovered type 10/12 streptococci in 2 of 10 samples from this company's barrack.

The distribution of bunks occupied by men yielding positive cultures for streptococci within the barrack is shown in figure 2. It was evident that there were cultures for type 1 and the one admitted patient were billeted in section 3 among the bunks sleeping 8 men. The distribution of type 10/12 was more random in the upper dormitory although a distinct focus was present in section 1. Ten of the 12 men from whom type 10/12 was recovered were billeted in the upper dormitory.

DISCUSSION

The experience of these two com-

panies of recruits in mid-February represented the first definite focal outbreaks of streptococcal infections during the winter from the regiment of 3200 men. Prior to this time, type 18 streptococcus had been the prevalent type isolated from admitted patients, and there had been no apparent concentration of infections in any unit or barracks. Types 1, 3, 5, 19 and 24 had also been sporadically isolated from admitted cases and carriers but had shown no ability to precipitate local outbreaks of infection. In contrast, type 10/12 streptococci disseminated rapidly and widely throughout the regiment following its appearance in companies A and B and accounted for 57% of all streptococcus-positive nose and throat cultures in the regiment during the month of February.

Observations at the time of the earlier outbreak of acute respiratory infections in Company A in January showed only minor evidence of streptococcal concentration in any section of the dormitory. The second episode of respiratory infections in the company presented a distinct epidemiological pattern of spread in the dormitory, with several distinct foci being apparent. The dispersion of type 10/12 was probably accentuated by the earlier phase of acute respiratory disease, in which 6 of 31 admissions and 2 nasal carriers harbored this strain of streptococci. During the second episode this was the only type isolated from admission cultures as well as on carrier survey.

Examination of the sleeping spaces occupied by men from whom streptococci were isolated either during carrier survey or upon admission revealed focal concentrations of positive throat cultures in each of the four sections of the lower dormitory and in two sections of the upper dormitory (fig. 1, b). The dissemination of streptococci appeared to follow the lower bunks in some areas, and again, to spread among men sleeping in upper bunks, possibly depending upon the billet occupied by the initial "disperser." A distinct relationship between nasal carriers and the occurrence of foci of positive cultures was not apparent in this pattern. It was also of interest that, despite the presence in the upper dormitory of 5 men who showed type 10/12 streptococci in their noses on admission, of whom three were still positive at the carrier survey, the isolation of this type was limited to men from two sections of the dormitory. This concentration in men living in close proximity to each other and absence in men who lived across the dormitory would seem to indicate that air or dust contamination was a minor factor in the spread of streptococci in these outbreaks. In Company B (fig. 2) this same pattern of spread is again apparent among men in adjacent bunks, although there is also a random dispersion of the different types through the various sections of the dormitory. The proximity of sleeping billets in these units of recruits apparently provided excellent facility for the spread of streptococci. The bunks were of the double-deck type and spaced with intervals of three feet between adjacent bunks and a space of five feet across the midline of the dormitory between the two rows of bunks.

The pattern in which streptococci were isolated from admitted cases and carriers in these two companies would strongly suggest that the greater virulence of the type 10/12 strain of streptococci, as compared to the other types present, was the most important determining factor in the occurrence of clinically recognizable focal outbreaks of streptococcal infections within the regiment. In Company A, the second outbreak consisted, for the most part, of streptococcal infections due only to type 10/12; the two other types present

earlier had disappeared completely. In Company B, 25 of the 30 men (83%) yielded types other than 10/12 at carrier survey, yet 10 of the 15 men (66%) admitted to the dispensary with positive cultures yielded this type from their throats. These men had all been diagnosed as having exudative tonsillitis or pharyngitis. Of the 6 men yielding positive cultures from the nose upon admission, type 10/12 was isolated from 5 and type 18 from one. Type 3 streptococci was isolated from 13 (43.3%) of the cultures positive at carrier survey, and two of these also had positive nose cultures, yet only two (13.3%) of the 15 admissions yielded this type. Type 1 was isolated from 23.3% of the carriers but from only 6.8% of the admissions, whereas type 18, although isolated from only 7.5% of the carriers, was recovered from 13.3% of the admissions. These findings would indicate that although other types of streptococci apparently spread with equal or greater facility than did type 10/12 in this company, they lacked the capacity to cause important clinical illness.

In clinical observations on more than 2000 recruits admitted to a dispensary for acute respiratory disease during the winter of 1947–48, the usual interval between the onset of illness and admission was two to three days. This interval, during which the individual may be presumed to be in a highly communicable stage of his disease, was spent in the dormitories or elsewhere with his company group. Those individuals who harbor streptococci in their nose or throat during this interval and who are capable of dispersing them into their environment would certainly appear to present a hazard of considerable magnitude. The possible role of these cases in the contamination of barracks and classrooms with spread of disease to their fellows suggests a pressing need for a satisfactory method of individual prophylaxis or at least early detection and removal of such cases from their units.

The incidence of acute respiratory disease observed in these two companies of naval recruits represents a common experience among such groups during the winter months, particularly following periods of increased recruiting.

SUMMARY

The experience of two companies of naval recruits which suffered outbreaks of acute respiratory infections associated with clearly defined foci of streptococcal spread within their dormitories is reported. Forty-one percent of the men were hospitalized for their illness during their first 8 weeks of training.

The disease pattern presented by these two companies was distinctly different. The first company suffered an outbreak of primarily nonstreptococcal respiratory disease immediately after entering the regiment. Type 10/12 streptococci was recovered from the throats of 6 men on admission and from the nose and throat of 2 other men upon carrier survey. Eight days later an outbreak, primarily streptococcal in origin, occurred, during which 11 admissions were positive for type 10/12 streptococci and 8 of the 11 also had positive nose cultures. Twenty-eight carriers of this type were found in focal concentrations throughout the barracks following the outbreak, and no other types were found.

The second company of 115 men had a single outbreak occurring three weeks after entering the regiment, during which there were 40 admissions. Ten of the 15 positive admission cultures yielded type 10/12 streptococci. Three other types were recovered from the remaining five admissions and, upon carrier survey at the end of the epi-

demic, 2 of these were more prevalent than type 10/12. The pattern of recovery of streptococci in barracks inhabitants demonstrated that these other types spread with at least equal facility among the carriers, focal concentrations being quite apparent, but lacked ability to produce clinical infections to any great degree.

The predominance of this one specific type of streptococcus in clinical illness, in nose cultures and in the air of the barracks of both companies, with at least two other types dominating the throat carriers in one company, presented an interesting variation in the communicability and degree of virulence of the several prevalent types of streptococci.

The focal concentrations of men yielding positive cultures for streptococci in the several sections of the dormitories suggested that the proximity of bunks provided excellent opportunity for the spread of streptococci between men in adjacent bunks. Outbreaks of streptococcal infections in these groups would appear to be favored by several epidemiological factors, most important of which were (1) the introduction of virulent strains by "dispersers," (2) the susceptible populations, (3) the two or three days between onset of illness and admission, during which a man remains with his unit and adds to the contamination of his environment and (4) the failure of the usual dispensary treatment schedules to effect a bacteriologic cure, allowing return of potential "dispersers" to their old or to new units.

A PNEUMOTROPIC PASTEURELLA OF LABORATORY ANIMALS. I. BACTERIOLOGICAL AND SEROLOGICAL CHARACTERISTICS OF THE ORGANISM

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Many laboratory animals are known to harbor infectious agents which produce spontaneous disease or are present in a latent state. Swiss mice have been studied with special care in this respect, and a number of bacteria[1–7] and other organisms[8,9] have been found to produce epizootic outbreaks from time to time in breeding colonies. Increasing interest in human respiratory infections has led to many attempts to adapt agents causing such diseases to small laboratory animals for more convenient study. Blind passage of lung material by the respiratory route under ether anesthesia frequently led to the production of lung lesions in the experimental animals due to the activation of latent agents. A number of latent viruses[10–16] and pleuropneumonia-like organisms[17–20] have consequently been found in apparently normal animals and have been studied in considerable detail.

As long as small laboratory animals are used for the study of infectious agents it will be important to have as complete an inventory as possible of latent organisms capable of producing disease on serial passage, together with a list of characteristics suitable for their rapid identification or elimination. This report deals with studies of a pneumotropic bacterium found widely distributed in colonies of laboratory animals. The organism is ordinarily latent, but it produces rather characteristic lung lesions on serial passage in Swiss mice, as reported in a preliminary note.[21] Several references to morphologically similar organisms were found in the literature,[2,22–24,31] but no attempt to

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evaluate their biologic and pathogenic properties has been recorded. It is the purpose of the paper to establish the characteristics of this bacterium.

MATERIALS AND METHODS

Experimental animals.—For most studies reported here albino Swiss mice were used. The animals were raised in the breeding colony of the National Institutes of Health. It was found early in the work that mice over 4 to 5 weeks of age were relatively resistant to the disease, and therefore 9 to 14 g mice 18 to 24 days of age were used in the majority of experiments. The animals were weaned at 17 to 19 days and fed on Purina Dog Chow pellets supplemented by small daily amounts of kale. Spontaneous disease was exceedingly rare in this stock, the normal, over-all mortality being always below 0.4%, although the majority of animals carried the pneumotropic organism to be described in their respiratory tract. In the past mice of this colony have been demonstrated[25] to harbor both PVM[12] and Nigg[13] viruses, but no evidence of these agents was encountered during the present study. The probable explanation is the rapidity of the transfers carried out at 2 to 4 days. Spontaneous pulmonary consolidation was observed in less than 1% of the stock animals (10,000 animals in stock, ca. 1000 surveyed), and no lesions resembling the experimentally produced acute consolidations were ever seen.

Serial animal passage.—Groups of 2 to 3 mice were killed by the intravenous injection of air. The lungs were removed aseptically, pooled and ground in a mortar with sterile alundum. Sufficient horse meat infusion broth was added to make a 10% suspension. On some occasions the livers and spleens of animals were similarly pooled and ground. Following light centrifugation (1500 r.p.m. for 5 minutes), the supernatant was administered intranasally to groups of 6 mice under ether anesthesia, 0.03 ml being instilled at an interval of 15 to 30 minutes. This double intranasal inoculation resulted in much more reliable and reproducible results than a single instillation.

Bacteriology and serology.—Cultures were grown routinely on 5% sheep blood meat infusion agar plates, and well isolated colonies were obtained by smearing infected lung over a small segment of the plate and streaking the remainder of the plate from that inoculum. Plates were incubated for 18 hours at 37 C. For purposes of bacteriological study the official methods[26] were followed.

Antigens were prepared from the various strains of organisms in the following manner: Cultures were grown on plain agar in Blake bottles at 37 C for 24 hours. The growth was washed off with 0.85% salt solution containing 0.1% formalin. The suspension was well shaken with glass beads, adjusted to a density of approximately 1 billion organisms/ml and stored at 4 C. Antiserums were prepared in rabbits weighing 3000 to 3500 g by the intravenous injection of increasing amounts of such antigens (0.1 ml to 1.0 ml) three times weekly for three weeks. The titer of the resulting serums ranged from 1:640 to 1:2560 in agglutination tests with the homologous antigens. These titers could not be increased significantly by further injections with either live or killed cultures. Other rabbits were inoculated with living cultures intraperitoneally and intravenously at weekly intervals. It was necessary to keep the dose below 1 ml of broth culture containing about 10^8 live organisms because that amount had a marked toxic effect. Serum titers after injection of 0.5 ml broth culture weekly for 5 to 6 weeks were lower than those obtained with formalin-killed antigens.

Agglutination tests were performed by adding to serial dilutions of serum in 0.2 ml amounts an equal quantity of antigen in 8×75 mm tubes. The tubes were shaken well and incubated for 1 hour at 37 C and 4 hours at 4 C. Readings were taken at the end of this period and again after the tubes had stood at room temperature overnight. Various other technics were tried but gave less satisfactory results. If the serial dilutions were prepared in normal rabbit serum instead of saline, the readings were ordinarily one to two twofold dilutions higher.

For agglutinin absorption the serum was mixed with 9 volumes of antigen suspension and incubated for one hour at 37 C. The cells were then centrifuged out and the absence of homologous agglutinins in this 1:10 dilution of serum determined. In several instances it was necessary to repeat absorption once, without further dilution, by adding packed bacterial cells and proceeding as before, in order to remove all homologous antibodies.

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Virulence titration.—Titration of passage strains was carried out with broth cultures started with a massive inoculum and incubated for 6 to 8 hours. Such cultures always contained between 5×10^7 and 5×10^8 organisms/ml by plate count. Serial dilutions of broth cultures were made in horse meat infusion broth just prior to inoculation. The test animals received two intranasal doses of 0.03 ml each under ether anesthesia, with an interval of 15 to 45 minutes between inoculations. In some experiments various laboratory animals were inoculated by other routes. All animals were observed for 14 days and the deaths recorded. At the end of this period the animals were sacrificed, autopsied and the lesions recorded and cultured. Lungs were recorded as "positive" if typical lesions were present and cultures revealed the presence of pasteurella. No attempt was made to evaluate the number of focal lesions or lobes involved. The results are reported in three ratios: (a) deaths/total, i.e., number of deaths divided by number of total animals inoculated, (b) lesions/survivors, i.e., number of animals with lung lesions over number of survivors, (c) infected/total, i.e., sum of deaths and survivors with lung lesions divided by total number of animals inoculated.

Serum protection tests.—Twenty-five hundredths ml serum was injected intraperitoneally 30 minutes prior to intranasal infection in the usual fashion. Animals were observed for two weeks and deaths recorded, the diagnosis being confirmed by autopsy and culture. Mouse serums for protection tests were obtained from animals which had survived infection and were bled 3 to 4 weeks later.

Antibiotics.—Sensitivity to antibiotics was determined by inoculating 0.1 ml of 18-hour broth cultures into tubes of meat infusion broth containing measured amounts of penicillin or streptomycin. These tubes were incubated for three days at 37 C and the broth subsequently streaked on blood agar plates to determine the presence of viable organisms. Unless turbidity of the broth indicated growth, the agar plate subcultures usually also remained sterile. The crystalline sodium salt of penicillin G was obtained commercially and dissolved in saline just prior to use. The streptomycin was kindly supplied by Dr. H. Welch, Food and Drug Administration, Washington D. C. in lots of 20 ml. The solution had been assayed by the Food and Drug Administration at 200 mgm/ml streptomycin base and was kept at 4 C for periods up to two months without loss of potency. The streptomycin solution was made up in suitable dilutions in normal saline and was either mixed with infectious lung material or injected subcutaneously into mice.[27]

Survey of animals for the presence of the agent.—Animals were obtained from various breeding stocks. When received at the laboratory, to prevent any possible cross infection, they were killed immediately by intravenous injection of air and autopsied. The lungs were removed with aseptic precautions and ground individually with alundum. Liver and spleen were ground together, and sufficient meat infusion broth was added to make 30 to 40% suspensions. Three or four loopfuls of such suspensions were streaked on blood agar plates and inoculated into broth tubes. All mediums were incubated at 37 C for 18 hours. The broth was plated as soon as growth became apparent. Suggestive colonies were picked from the plates and identified by the usual morphological, cultural, biochemical and serological criteria. The findings are reported as positives/total, i.e., the ratio of animals harboring pasteurella to the total number of animals sacrificed.

EXPERIMENTAL

The experimental disease of mice

In the course of attempts to adapt to Swiss mice an agent causing a minor respiratory illness in human volunteers[27] pneumonic lesions were encountered in animals inoculated intranasally under ether anesthesia with mouse lung material in serial passage. In early passages only a transient illness with lassitude, anorexia and ruffled coat occurred between the 6th and 10th day after inoculation, and there were no definite lesions in any organs of the mice killed at that time. Starting with the 5th passage the illness was more acute and definite, though usually not fatal, and the pathological findings were limited to clear-cut grayish consolidation of the upper lobes. In the 7th passage there were a few deaths between the 3rd and the 15th day after inoculation. The proportion of deaths rapidly increased so that in the 14th passage of mouse lung material 85% of the animals died after an illness of 2 to 7 days.

The clinical picture of the disease at

27. Topping, N. H. and Atlas, L. T. 1947, Science, **106**: 636.

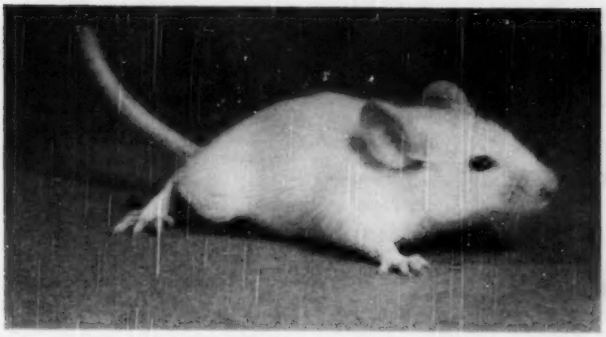

FIG. 1.—Mouse appearing quite healthy, 3 hours after intranasal inoculation with 10^6 organisms from the 16th passage *P. pneumotropica* culture.

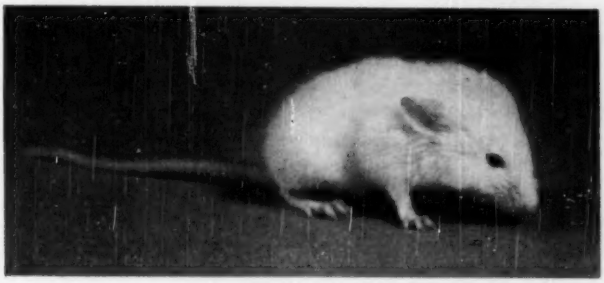

FIG. 2.—Same mouse 28 hours after inoculation.

FIG. 3.—Same mouse, moribund, 92 hours after inoculation.

that stage was rather constant.[21] About 12 to 18 hours after intranasal inoculation the mice became inactive, lethargic and hunched, with ruffled fur (fig. 1 and 2). Later breathing was labored and rapid, the mice refused food and water, and occasionally conjunctival exudate glued the lids together. Twenty-four to 48 hours after inoculation distinct "chattering" was heard, the dyspnea was more severe, and cyanosis of ears, tail and feet was apparent. These

symptoms progressed with gasping respiration and deep cyanosis (fig. 3) to a fatal outcome after 2 to 7 days in the majority of animals, while others were not quite so ill and ultimately recovered clinically.

The first gross pathological changes were observed 18 to 24 hours after infection when the lungs assumed a mottled appearance. Somewhat later definite foci of reddish-grey bronchopneumonia appeared in all lobes, predominantly close to the hilum. Forty-eight to 72 hours after infection the consolidation progressed to form sharply delimited grey foci, often occupying much of one lobe and found most commonly in the left upper and the azygos lobes. Later yellowish centers of necrosis became visible within the zones of grey consolidation. If the animals survived, the grey, pneumonic zones gradually regressed, while the necrotic areas became surrounded by a firm fibrous capsule and the contents of this nodule became homogenous, yellowish-white and viscid. Such nodules could be found two months after infection in otherwise normal appearing lungs, when they protruded spectacularly from the surface.[36]

The histological picture was one of a mixed polymorphonuclear and mononuclear exudate in the lungs, filling alveoli and bronchi in the early stages, with subsequent central coagulation necrosis of the consolidated areas. All other organs were essentially normal. A more comprehensive description of the experimentally induced lesions in both the progressing and the regressing stages is presented in the accompanying paper.[36]

Studies on etiology

1. Recovery of organisms.

Smears from consolidated lung areas showed many minute extracellular gram-negative rods which could be stained brilliantly red by Macchiavello's method. From the lesions a short coccoid bipolar gram-negative rod was cultivated regularly on simple mediums. Occasionally there were also larger, gram-negative, lactose-fermenting rods present in the lungs, and sometimes α-hemolytic streptococci and diphtheroids were found.

In the earlier passages the disease could not be reproduced by pure cultures of any of the organisms grown on artificial mediums. Berkefeld V and Seitz filtrates of 10% infective lung tissue likewise had no effect. No evidence could be found for the presence of viruses of any type, many stains for elementary bodies were negative,[18] and mouse red cells were not agglutinated by lung suspensions heated at 70 C for 30 minutes.[12,28]

The cultures of pasteurella-like organisms recovered from early passages were apparently not pathogenic. From the 14th serial mouse passage, however, cultures of this organism were isolated which quite regularly reproduced the disease on intranasal inoculation and resulted in a mortality of 85%. In the 16th mouse passage the LD_{50} of the pure culture[29] reached 5×10^5 organisms, given intranasally, while the comparable end point for infectivity (ID_{50}) measured by the presence of lung lesions was 10^4 bacteria.

Virulence of such cultures was rather unstable. Repeated transfer on artificial mediums soon resulted in partial loss of pathogenicity and even cultures frozen at −50 C or −72 C or kept in the lyophilized state did not preserve their peak virulence. Frequent animal passage was essential to maintain strains of

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maximal pathogenicity. The following routine sequence was adopted for virulent test cultures: Lyophilized culture, broth culture, mouse passage, lungs plated on blood agar, pure culture on blood agar, colonies picked into broth for challenge culture. While peak virulence could be maintained only by frequent animal passage, daily transfer of broth cultures did not result in complete loss of pathogenicity. Thus after 18 daily broth transfers the culture still produced a few deaths and lesions, though with a much prolonged incubation period.

TABLE 1.—*Biochemical characteristics of strains of* P. pneumotropica.

Organisms	Broth	Potato slant	Gelatin liquefaction	Indol production	Nitrate reduction	H_2S production	Methyl red test	MacConkey's agar	Litmus milk	Acid but no gas from: Adonitol	Arabinose	Dextrose	Glycerol	Inositol	Lactose	Maltose	Mannose	Rhamnose	Salicin	Sorbitol	Sucrose	Xylose
M 10	Turbid	–	–	+	+	–	–	–	Neutral	–	+	+	+	+	+	+	+	–	–	–	+	–
M 177	Sedim.	–	–	+	+	+	–	–	Neutral	–	+	+	+	–	+	+	+	–	–	+	+	–
M 178	Turbid	–	–	+	+	–	–	–	Neutral	–	–	+	+	+	+	+	+	–	–	–	+	–
M 182	Turbid	–	–	+	+	–	–	–	Neutral	–	–	+	+	+	+	+	+	–	–	–	+	–
BS 5	Turbid	–	–	+	+	–	–	–	Acid	–	–	+	+	+	+	+	+	–	–	–	+	–
BS 3	Turbid	–	–	+	+	–	–	–	Acid	–	–	+	+	+	–	+	+	–	–	–	+	–
BS 2	Turbid	–	–	+	+	+	–	–	Alkaline	–	+	+	+	+	+	+	+	–	–	+	+	–
CFW 6	Turbid	–	–	+	+	–	–	–	Neutral	–	–	+	+	+	+	+	+	–	–	–	+	–
CFW 9	Turbid	–	–	+	+	–	–	–	Neutral	–	–	+	+	+	+	+	+	–	–	–	+	–
Ca 2	Turbid	–	–	+	+	–	–	–	Alkaline	–	–	+	+	+	+	+	+	–	–	–	+	–
Ca 6	Turbid	–	–	+	+	–	–	–	Neutral	–	–	+	+	+	+	+	–	–	–	–	+	–
Che	Turbid	–	–	+	+	–	–	–	Neutral	–	–	+	+	+	+	+	+	–	–	–	+	–

2. Bacteriological characteristics of the organism.

Morphology.—The organism was a short, occasionally coccoid, bipolarly stained, gram-negative rod, measuring 0.4 to 1 × 0.6 to 2 μ, nonmotile and nonsporeforming. An indistinct envelope was observed with negative staining. In older cultures there was marked pleomorphism, with threadlike and swollen forms.

Growth.—There was abundant growth on simple mediums, including plain nutrient and meat infusion agar and broth, rapid at 36 to 37.5 C and slower at 22 C. Aerobic conditions were preferred, but there was some proliferation also under strictly anaerobic conditions both in liquid and solid mediums. In meat infusion broth there was uniform turbidity after 6 to 18 hours at 37 C; later sometimes a pellicle was formed and sediment deposited. On solid mediums 18-hour colonies were convex, whitish, round with entire edge, about 1 mm in diameter, with smooth surface. No hemolysis was produced in mediums containing sheep or rabbit blood. No growth appeared on potato slants, MacConkey's or Desoxycholate agar.

Biochemical activity.—All strains showed a uniform pattern of metabolic activity, as shown in table 1.

The bacterium showed somewhat unusual survival characteristics. Cultures in liquid mediums remained viable much longer at room than at refrigerator temperature, while on solid mediums the reverse was the case (table 2). It has been mentioned already that storage at −50 C or in the lyophilized state preserved viability but not maximal virulence.

On the basis of morphological, cultural and biochemical characteristics the organisms must be classified as a pasteurella, resembling *Pasteurella multocida*.

Serology.—Agglutination tests were carried out with antigens from 14 strains

of the mouse pasteurella against homologous and heterologous antiserums. The mouse pasteurella antigens were agglutinated to titers of 640 to 2560 by antiserums against the homologous strain, and to titers of 320 to 2560 by antiserums against heterologous strains (table 3). These antigens were not agglutinated by antiserums against *Pasteurella pestis*, *Pasteurella pseudotuberculosis*,*, *P. multocida*, *B. bronchiseptica*, *Hemophilus influenzae* type B, or *Hemophilus pertussis* phase I.

Cross absorption tests with antigens representative of three groups of strains of the mouse pasteurella (table 3) indicated that there were some antigenic differences between groups of strains isolated from different animal colonies. However, strains from a given animal colony had a similar antigenic pattern.

Pathogenicity.—Three strains of mouse pasteurella were passed serially until virulence for respiratory inoculation was evident. With the 1st strain this happened at the 14th passage (85% mortality), with the 2nd after the 3rd passage (70% mortality), and with the 3rd after the 9th passage (90% mortality). On many occasions the relative virulence of the bacterium was tested by other routes of inoculation into susceptible mice. Intracutaneous, subcutaneous, intraperitoneal and intravenous injection failed to have any effect, while intranasal instillation of similar doses from the same culture resulted in deaths, or pneumonic lesions, of all inoculated mice (table 4). In 2 mice out of 60 injected with 3×10^7 viable bacteria by the intravenous route typical minimal pulmonary lesions were produced from which the organism was cultured. However, one tenth of this inoculum, instilled intranasally, killed the majority of animals. The parenteral injection of living organisms produced some immunity (table 4), although not giving rise to clinical disease or lesions.

Intracerebral injection of the pasteurella was the only route apart from the intranasal one regularly resulting in illness and death of mice. The pathology, however, was quite different. Essentially it resulted in formation of a brain ab-

TABLE 2.—*Viability of* P. pneumotropica *cultures under various conditions of storage.*

Culture medium	Storage temperature (Centigrade)	Last positive subculture obtained (days after inoculation)		
		Strains: M 193	M 10	M 196
Meat infusion broth	4	28	28	28
	20	49	29	>100
	37	20	24	28
Meat infusion broth with 5% sheep blood	4		> 90	56
	20	>100	>100	85
5% sheep blood agar slant	4	28	28	28
	20	12	20	20
Meat infusion broth	−50	>245	>245	>245
Skim milk	−50	>245	>245	>245
Skim milk (lyophilized)	4	>245	>245	>245

TABLE 3.—*Cross absorption-agglutination tests with* P. pneumotropica *strains.*

Antigen	Anti-M 10 serum unabsorbed	Anti-M 10 serum absorbed with		
		M10	M (CFW2)	M (Ca 6)
M 10	2560*	0	80	20
M 178	640	0	160	40
M 182	320	0	80	0
M (CFW 2)	640	80	0	20
M (CFW 6)	1280	160	0	20
M (BS 5)	1280	40	160	0
M (Ca 2)	1280	0	320	0
M (Ca 6)	2560	0	320	0

Origin of strains: M 10, M 178, M 182 from regular stock, National Institutes of Health mouse colony; M (CFW 2), M (CFW 6) from Carworth Farms, CFW mouse colony, New York; M (Ca 2), M (Ca 6) from National Cancer Institute mouse colony, Bethesda, Md.; M (BS 5) from National Institutes of Health, brother-sister mated mouse colony.

* Figure indicates highest dilution giving 4 plus agglutination.

* Tested through the courtesy of Miss J. B. Gunnison.

scess with typical neurological signs ending in coma and death in 36 to 72 hours. Ordinarily only brain cultures of such animals were positive, all other organs negative, indicating a localized process. The intracerebral injection of more than 10^6 organisms was invariably fatal, and the LD_{50} for this route was about 10^4 bacteria. Streptomycin treatment was strikingly effective in this form of infection, as described elsewhere.[37]

Many attempts were made to produce disease or lesions in laboratory animals other than mice. Injection by various routes of white rats, cotton rats, hamsters, guinea pigs, rabbits and chickens resulted in failure. On several occasions suggestive small pneumonic areas were encountered in the lungs of guinea pigs and cotton rats receiving large intranasal inoculums, but cultures failed to reveal the pasteurella. No proof of infection of any of these animal species could be obtained after inoculation by any route.

It appears that this pasteurella is very highly adapted to the respiratory tract of laboratory animals, particularly mice, retaining its pneumotropic character even with increased pathogenicity after rapid animal passage. Consequently the organism is referred to as *Pasteurella pneumotropica*.

3. Immunization.

If the mouse pneumonia were entirely due to pasteurella infection, immunization against this organism should protect the animals against the disease. A suitable experiment is shown in table 4. Mice 18 to 21 days old and weighing 10 to 12 g were given virulent broth culture of the 17th passage by various parenteral routes, or formalinized suspensions of the same passage strain. All but one of the control animals receiving the culture intranasally succumbed, while parenterally injected mice tolerated the inoculum well. Ten days later the animals were challenged with an intranasal dose of virulent pasteurella culture, together with suitable controls of the same age and weight. Fifteen of 16 control mice died, while the majority of previously injected animals resisted the infection. A single dose of live virulent pasteurella apparently resulted in more substantial immunity than the much larger number of formalin-killed organisms.

TABLE 4.—*Effect of parenteral administration of live and killed* P. pneumotropica *on the results of subsequent challenge with the same strain, in mice.*

First administration of *P. pneumotropica*					Results of intranasal challenge		
Route	Dose (ml)	Material	Number of organisms	Death/ total	Death/ total	Lesions/ survivors	Infected/ total
I.N.	0.06	Virulent broth culture	10^7	15/16	0/1	0/1	0/1
S.C.	0.2	Virulent broth culture	3×10^7	0/16	4/16	3/12	7/16
I.P.	0.2	Virulent broth culture	3×10^7	0/16	2/16	2/14	4/16
I.V.	0.06	Virulent broth culture	10^7	0/16	1/16	2/15	3/16
I.V.	0.1	Formolized suspension	10^8	0/16	6/16	1/10	7/16
I.P.	0.1	Formolized suspension	10^8	0/16	5/16	4/11	9/16
		None—Controls		0/16	15/16	1/1	16/16

I.N.—intranasal inoculation under ether anesthesia.
S.C.—subcutaneous injection.
I.P.—intraperitoneal injection.
I.V.—intravenous injection.
Other symbols as explained in text.

Inoculation with an avirulent live *P. pestis* culture[30] failed to protect against challenge infection with *P. pneumotropica*. The results of the active immunization experiments were com-

30. Jawetz, E. and Meyer, K. F. 1943, J. Infect. Dis. **73**: 124.

patible with the hypothesis that the mouse pasteurella alone was responsible for the mouse disease under study.

4. Antibody response and action of hyperimmune serum.

To confirm the etiologic relationship between the pasteurella cultures and the observed mouse pneumonitis, serological studies were attempted on mice that had survived the infection. Groups of such mice were bled at different times after clinical recovery and the serums pooled and tested for agglutinating antibodies against the homologous antigens. By this technic antibodies could be demonstrated only in very low titers, never exceeding 1:16, rarely 1:4. The same mouse serums were also tested for protective antibodies and compared to serums prepared in rabbits. Convalescent serums did not afford protection in these experiments, and even undiluted hyperimmune serum (with agglutination titer of 1:2560) had only moderate protective power.

5. Effects of antibiotics.

Cultures of the pasteurella showed a uniformly high sensitivity to streptomycin so that growth was completely inhibited in broth by 2 to 4 μg/ml. Penicillin resistance was always high, and from 50 to 100 units/ml were necessary for complete inhibition of growth in liquid mediums. It has been pointed out elsewhere[21] that mixing infective lung suspensions with streptomycin prior to intranasal instillation effectively controlled transmission of the pasteurella. This method can be used effectively for the control of the organism in the course of intranasal passage of virus-containing material.[21,24]

Streptomycin treatment of animals infected by the intracerebral or intranasal route was likewise highly effective.[37] These results suggested that a streptomycin-sensitive organism was in part at least responsible for the illness and death of the mice.

Incidence of P. pneumotropica *in various animal colonies*

All evidence supported the belief that the pasteurella recovered from lung lesions of passage mice was the etiologic agent responsible for the observed disease and not merely an incidental saprophyte. It was important to determine whether such organisms were present only in the one mouse colony from which they were originally recovered or whether their distribution was more widespread. Consequently, representative groups of animals were obtained from other breeding stocks and screened for the presence of the agent by culturing suspensions of organs.

The organism was recovered by simple bacteriological means not only from three different breeding colonies housed in the same building as the mouse stock found infected in the beginning of this work, but also from several other mouse colonies (table 5). In fact, among the 5 different breeding establishments surveyed in the eastern United States not a single one was found free from the pasteurella. Most commonly the organism was cultured from lung suspensions, but occasionally it was also recovered from spleen and liver suspensions enriched in liquid medium. The variations in the frequency with which the pasteurella was isolated from different stocks (table 5) probably does not reflect the true incidence of carriers. Careful inquiry into the past history of the mouse colonies especially with respect to epizootic disease revealed occasional intermittent outbreaks of enteritis but of no other sporadic or epizootic disease. This suggests that the pasteurella is entirely latent under ordinary conditions and that virulence is only

acquired, or enhanced, when serial lung passages are carried out or host resistance declines sharply.[31]

Examination of guinea pigs, white rats, cotton rats and hamsters reared in facilities adjacent to a known infected mouse colony gave positive results. The pasteurella was cultured from the respiratory tract but no other organs of these animals. No animals from other stocks or breeders were examined.

pears to be entirely latent in the respiratory tract of laboratory animals. It produces disease only under the artificial circumstances of serial transfer of lung material or possibly drastic reduction of host resistance.[32] In none of the mouse colonies infected with *P. pneumotropica* has there been an outbreak of respiratory disease or hemorrhagic septicemia for a number of years. Contact experiments[30] did not result in trans-

TABLE 5.—*Incidence of* P. pneumotropica *infection in various breeding stocks of small laboratory animals.*

Animal	Colony	Positive/total*	Percent positive
White mice	Natl. Inst. Health BS	8/20	40.0
White mice	Natl. Inst. Health Reg. adults	5/10	50.0
White mice	Natl. Inst. Health CFW	3/10	30.0
White mice	Natl. Inst. Health Reg. 7 days old	6/14	42.8
White mice	Natl. Cancer Inst.	10/10	100.0
DBA mice	Natl. Cancer Inst.	6/8	75.0
White mice	Research Laboratory #1†	7/16	43.7
White mice	Commercial breeder, New York State (CFW)	11/16	75.0
White mice	Research Laboratory #2‡	1/12	8.3
Guinea pigs	Natl. Inst. Health	3/8	37.5
White rats	Natl. Inst. Health	7/8	87.5
Cotton rats	Natl. Inst. Health	2/8	25.0
Hamsters	Natl. Inst. Health	1/6	16.6

* Positive animals by culture divided by total number of animals autopsied.
† Through the courtesy of Dr. J. E. Smadel.
‡ Through the courtesy of Dr. F. S. Cheever.

DISCUSSION

Pasteurellosis of laboratory animals is a common disease and has been noted in the past especially in epizootic outbreaks of hemorrhagic septicemia in breeding colonies.[1,3–7] In experimental epidemiology[5–7] such epizzotics provided a convenient tool for the study of the behavior of parasites and hosts under controlled conditions. The etiologic agent, *P. multocida*, is usually transmitted by the respiratory route and produces disease only in a portion of the infected animals, remaining latent in the others. When spontaneous illness occurs it ordinarily takes the form of a fulminating septicemia.

The organism described here presents somewhat different characteristics. Under natural conditions it appears to be entirely latent in the respiratory tract of laboratory animals. It produces disease only under the artificial circumstances of serial transfer of lung material or possibly drastic reduction of host resistance.[32] In none of the mouse colonies infected with *P. pneumotropica* has there been an outbreak of respiratory disease or hemorrhagic septicemia for a number of years. Contact experiments[30] did not result in transmission of disease in spite of apparently optimal conditions and the presence in the mouse population of highly virulent strains of the bacterium.

The bacterium is furthermore quite unusual in its affinity for tissues of the respiratory tract. Its pneumotropism is so marked that even after intravenous injection of large doses the organism fail to persist and produce lesions anywhere except occasionally in the lungs.

For purposes of classification the data presented here warrant the conclusion that the described organism is a pasteurella. Among related bacteria the *Hemophilus* group is set apart by its growth requirements and lack of bipolar staining; *B. bronchiseptica* by its lack of carbohydrate fermentation with acid production and motility; and *P.*

31. Carworth Farms Quarterly Letter, April 1, 1949.

32. Rosenbusch, C. T. and Merchant, I. A. 1939. J. Bact. **37**: 69.

pestis, *P. pseudotuberculosis*, *Pasteurella tularensis* and *Pasteurella hemolytica* are differentiated by their different antigenic structure, biological characteristics and pathogenicity. There are at least three serological groups and perhaps more of *P. multocida*,[32,33] and a possible antigenic relationship to the mouse pasteurella cannot be ruled out on the basis of available data. However, most *P. multocida* strains have different biochemical reactions and exhibit striking sensitivity to penicillin.[34,35] Virulent *P. multocida* pathogenic for many experimental animals but not for the natural host have been found latent in the throat of wild rats in Baltimore.[34] In the case of *P. pneumotropica* the situation seems reversed in that pathogenicity is apparently limited to one of the natural hosts and only under artificial circumstances.

To establish the relationship of *P. pneumotropica* to the lesions from which it was isolated, Koch's postulates have been fulfilled. The isolation of the organism from all lesions, growth in pure culture, and production of similar lesions with such cultures do not constitute absolute proof that the bacterium is solely responsible for the effects observed. A possible association of the pasteurella with some other agent, present constantly and latently in the experimental animals could not be ruled out. It is certain, however, that the pasteurella is essential for the production of disease and lesions in all cases, and that no evidence of any other agent has been found.

It is of interest that somewhat similar bacterial have been reported in the past. Andrewes et al[22] noted that mice inoculated with influenza viruses occasionally yielded a small bipolar gram-negative bacillus, which was considered "of no significance." Hoyle[3] observed among bacteria recovered from mice "gram-negative coccobacilli" resembling *H. influenzae*, but growing on plain agar. In the course of virus passages in animals these bacilli increased in numbers and virulence so that 0.5 ml of broth culture often killed the mice, whereas intranasal instillation did not. Kairies and Schwartzer[23] noted "spontaneous influenza" among their mice "precipitated by cold wet air." They recovered an organism resembling *H. influenzae* but growing readily on simple mediums. The same bacteria were found in 35% of normal mice and in 50% of mice inoculated with influenza material. A spontaneous epizootic was observed, and bacteria of increased virulence killed mice on subcutaneous or intraperitoneal inoculation.

A study of these reports leaves little doubt that the various organisms should be grouped as pasteurella. Comparison with the *P. pneumotropica* described in this paper leads to the conclusion that they are related to but not identical with it.

The various pasteurella-like organisms occurring in the respiratory tract of rodents might be arranged on a gradient. At one end would be placed *P. multocida*, an invasive organism of potentially high virulence by any route of administration, at the other end *P. pneumotropica*, with highly specific tissue affinity and low invasiveness and virulence. The organisms reported by Hoyle et al and others would occupy intermediate positions. All these agents

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33. Roberts, R. S. 1947, J. Comp. Path. & Therap. **57**: 261.
34. Schipper, G. H. 1947, Bull. Johns Hopkins Hosp. **81**: 333.
35. Needham, G. N. 1948, Proc. Staff Meet. Mayo Clin. **23**: 361.
36. Jawetz, E. and Baker, W. 1950, J. Infect. Dis. **86**: 184–196.
37. Jawetz, E. 1948, Proc. Soc. Exper. Biol. & Med. **69**: 105.

might stem from a common ancestor, and their characteristics might be the result of gradual adaptive changes in both host and parasite.

With the full realization that the organism described here represents merely a point on a sliding scale, the descriptive name of *P. pneumotropica* is proposed for the sake of convenience. However, the dynamic processes of biologic adjustment are in constant flow, and recognition of movement and change must remain more important than the arbitrary fixation of characteristics at any given point.

SUMMARY

1. In the course of rapid serial passage of mouse lung, pneumonic lesions were encountered. A pasteurella-like organism was regularly recovered from the lesions and shown to be responsible for the disease process.

2. Bacteriological and serological studies indicated that the pasteurella was not identical with any known species. Its outstanding characteristic was its striking pneumotropism in mice, producing no lesions in any organ except the lung after inoculation by any route. Therefore the name of *Pasteurella pneumotropica* is proposed for this bacterium.

3. Mice from a number of mouse colonies in the eastern United States were shown to carry the organism latently in their respiratory tract. No spontaneous epizootics have been noticed in these colonies for several years. Pathogenicity of latent *P. pneumotropica* appears to be a function of intranasal passage of mouse lung material. The organism was also recovered from the respiratory tract of other small laboratory animals, but no lesions could be induced in them.

4. It is suggested that *P. pneumotropica* is related to other *Pasteurella* species found in mice and that its peculiar characteristics may have evolved in the course of adaptive changes in host and parasite.

A PNEUMOTROPIC PASTEURELLA OF LABORATORY ANIMALS. II. PATHOLOGICAL AND IMMUNOLOGICAL STUDIES WITH THE ORGANISM

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In the preceding paper a pasteurella has been described which differs from established species in several biological characteristics, particularly in its very marked pneumotropism in mice. Such pneumotropism is unusual in bacteria, and on the basis of past experience with other pasteurellas[1] it was thought that perhaps host resistance might account for it.

To study the dynamics of the infection it was necessary to investigate the distribution and spread of bacteria in the host and to relate the findings to the observed pathological changes. It was then important to establish the exact method of transmission of the ordinarily latent infection in a population and to determine, if possible, the relative role of inherent and acquired resistance to the experimental disease. Finding the pasteurella highly susceptible to streptomycin action suggested use of this antibiotic for studies in chemotherapy and for experiments designed to raise a stock of mice free from the latent infection.

This paper presents a detailed gross and microscopic study of the experimental disease caused by *Pasteurella pneumotropica*, supporting the previous contention that this bacterium has striking pneumotropic properties. Experimental evidence is produced to support the suggestion that the host resistance to the infection is inherently high and not the result of immunization by subclinical infection.

METHODS

Experimental animals.—Swiss mice bred in the colonies of the National Institute of Health were used for all studies. Most of these animals carry *P. pneumotropica* latently in their respiratory tract but present no evidence of illness.[2] Ordinarily "immature" mice (18 to 21 days old) were used, but in some instances "mature" animals (35 to 50 days old), as specified in the text. The care of these animals and their freedom from spontaneous disease has been described.[2]

P. pneumotropica.—The organism and culture methods employed have been described. For most studies virulent 15th to 20th mouse passage strains were used, commonly an 8-hour broth culture fron a sheep blood agar plate as necessary for maintenance of full virulence. Standard inoculation procedures were the same as used before.[2]

Pathologic study.—For the purpose of studying the pathogenesis of the lesions induced by the pasteurella, 50 21-day-old Swiss mice were inoculated by the intranasal route under light ether anesthesia with approximately 6×10^5 virulent organisms. The animals were observed and at intervals up to 26 days, groups of three were sacrificed. Twenty-two animals died during the experiment and were not examined. A total of 28 mice was studied pathologically with simultaneous culture. Sections were obtained routinely from lungs, liver, spleen, kidney, adrenal, heart, trachea and esophagus. Tissue was fixed in 10% formalin for 48 hours and stained by azure eosin and Van Gieson methods.

Bacteriological studies.—These were performed according to a technique described in detail elsewhere.[1] Essentially it consisted of sacrificing ani-

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1. Jawetz, E. and Meyer, K. F. 1944, J. Infect. Dis. **74**: 1–13.

2. Jawetz, E. 1948, J. Infect. Dis. **86**: 172–183.

mals at intervals and performing the autopsy with aseptic technique, using fresh sterile instruments for each organ. Standard cross sections of organs were smeared over segments of blood agar plates. The plates were incubated at 37 C and read after 24 and 48 hours. Typical colonies were counted and identified in the manner previously described.[3] The results are reported in the following terms: No pasteurella colonies present, −; 1 to 5 colonies, +; 5 to 20 colonies, ++; 20 to 50 colonies, +++; more than 50 colonies per standard segment of blood agar plate, ++++. The method is not quantitative but permits an evaluation of the progress of bacterial infection in animals of a series uniformly treated.

Hematology.—At varying intervals after intranasal infection, a group of mice was bled from the tail and total white blood counts made in the usual fashion. When the animals were moribund they were anesthetized with ether, the blood vessels in the axilla severed, and the blood drawn into a microhematocrit tube after suitable addition of anticoagulant. The tube was centrifuged at 2200 r.p.m. for one-half hour and the reading made.

Antibiotics.—The streptomycin used in this work was obtained through the courtesy of Dr. Henry Welch, Food and Drug Administration, Washington, D. C. in lots of 20 ml, standardized as 200 mg of streptomycin base per ml of solution by the Division of Penicillin Control of the FDA. The material was kept stored at 4 C for periods up to two months without loss of potency.

Raising of pasteurella-free mice.—For certain studies it was desirable to obtain mice free from latent pasteurella infection without any contact with infected animals. For this purpose the following method was used: Pregnant mice likely to have litters within one week were brought to the laboratory and started on subcutaneous injections of streptomycin, 5000 units per day in a single injection (approximately 0.15 g/kg/day). After two days of such treatment the pregnant mice were transferred to an isolation room, where cages, bedding, food and water had been sterilized and to which only two attendants had access. The animals were kept in single isolation cages and were not handled except for the daily injection of streptomycin, until the time of delivery, or the 5th day of isolation, whichever occurred sooner. After the litters were born no more streptomycin was administered, and the cages were not touched or changed until the 10th day of age. The offspring were kept in the isolation room until used at 18 to 21 days of age. Control mice were treated in exactly the same manner only the streptomycin injections and the isolation room were omitted.

EXPERIMENTAL

Pathologic findings

At regular intervals after infection groups of animals were sacrificed and the following changes observed:

Twenty-four hours.—The lungs showed spotty gray-red areas which were scattered throughout both lungs but were most pronounced at the hilus.

Microscopic (fig. 3A).—Throughout the lung there were small areas of predominantly peribronchial alveolar involvement. The alveoli in these areas were filled with an exudate of polymorphonuclear leukocytes, few large mononuclear cells and a small amount of fibrin. In addition, a few contained red blood cells and basophilic and pleomorphic short bacilli. Most bronchi were uninvolved but occasionally an exudate of polymorphonuclear leukocytes and mononuclear cells was present. The alveolar capillaries throughout the lung were congested.

Forty-eight hours.—The reddish-gray areas of consolidation had extended, involving about ⅓ of the lungs (fig. 1).

Microscopic (fig. 3B).—The small foci of alveolar involvement had coalesced. The exudate was similar to that seen at 24 hours. The alveolar lining cells were very prominent and cuboidal and many were desquamated. A few bronchi contained polymorphonuclear leukocytes and red blood cells.

Seventy-two hours.—Two of the animals showed well demarcated areas of grey consolidation involving the upper lobes of both lungs and the azygous lobe of the left. On cut section the surface was firm, gray and gelatinous. The third animal was entirely normal.

Microscopic.—The cells in the alveoli (polymorphonuclear leukocytes and mononuclears) exhibited pyknosis and karrhyorexis. There were large circumscribed areas of oxyphilic necrosis containing nuclear fragments and remnants of poorly staining alveolar walls. In adjoining alveoli there was some fibrin and many preserved cells. Most of the bronchi within the involved areas contained a polymorphonuclear exudate within their lumen.

Ninety-six hours.—There was no essential change in the gross or microscopic picture.

Five days.—There was extensive gray consolidation in the upper lobes of both lungs. Within the areas were several small, yellow nodules measuring 1 to 2 mm in diameter. They were well defined and on section contained a very viscid yellow-white fluid.

Microscopic (fig. 4A).—The cellular exudate within the alveoli showed more degeneration and many shed alveolar septal cells. Numerous abscesses were present, the centers of which showed coagulation necrosis. At their periphery there was a moderate amount of fibrin, and adjacent to it a thin rim of polymorphonuclear leukocytes. The surrounding alveoli were collapsed, and the bronchi in these areas contained an exudate of polymorphonuclear leukocytes.

Seventh day (fig. 2).—The lung lesions were well demarcated, gray-white, translucent and considerably smaller than similar areas noted before. In two of the animals the lesions were confined to the left upper lobe, and in the third mouse the apex of the right upper lobe was also involved. Within these areas of consolidation, yellow nodules were again seen, now better demarcated than before. The spleen was slightly enlarged.

Microscopic.—The lesion consisted of well circumscribed areas of coagulation necrosis as seen on the 5th day. Many alveoli between these areas were collapsed and a few contained a mixed cellular exudate. Elsewhere the alveoli were empty, and the interstitial tissue contained many polymorphonuclears and mononuclear cells.

The spleen in all three animals showed follicular hyperplasia with almost the entire follicle composed of reticulum cells showing active cytophagocytosis.

Ninth day.—Again the gray-white areas of consolidation were confined to the lung apices and contained nodular yellow areas of necrosis. The spleen was moderately enlarged. One mouse had a small, yellow-white nodule at the tip of the uterus which measured 6 mm in diameter and on section contained viscid yellow-white material.

Microscopic.—The microscopic changes in the lungs were the same as on the 7th day. The nodule above the uterus showed a center of coagulation necrosis surrounded by a well defined fibrous capsule. The same mouse showed, throughout the liver small, well circumscribed areas of liver cell necrosis, with replacement by polymorphonuclear leukocytes, fibrin and an occasional mononuclear cell. These abscesses had no definite lobular pattern but were most frequent in the pericentral areas.

Twelfth day.—The gray areas of consolidation at the apices were very small with relative increase in prominence of the yellow nodules. The spleens were slightly enlarged.

Microscopic (fig. 4B).—The well circumscribed areas of coagulation necrosis varied in size but showed a uniform histological picture. The central core of necrosis was surrounded by shreds of fibrin and a wide zone of polymorphonuclear leukocytes. Exterior to this layer there were numerous large pale endothelial cells which exhibited phagocytosis and contained vacuoles. These cells stained red on frozen section using Oil Red O. There was a capsule of proliferating fibroblasts and fibrocytes and a little collagen. Some adjacent alveoli were collapsed, and the uninvolved alveoli showed an increase in polymorphonuclear leukocytes in the interstitial tissue.

Nineteen and twenty-six days.—The lesions were confined to the left lung, where yellow circumscribed nodules were still present, while the remainder of the lung tissue had resumed its normal appearance. The spleens were moderately enlarged.

Microscopic.—The histological picture remained essentially unchanged. From the 1st to the 7th day after infection the tracheas of the mice showed a small number of polymorphonuclear leukocytes in the submucosa but no exudate in the lumen or destruction of the mucosa. The hilar nodes became enlarged at 24 hours and remained so throughout the entire experiment. The histological picture was that of chronic inflammation with follicular hyperplasia and active cytophagocytosis within the germinal centers.

Clinicopathologic correlation

A correlation of the gross pathological lesions with the clinical picture and results of bacteriological studies are presented in table 1. Twenty-four hours

FIG. 1.—Gross appearance of mouse lung 48 hours after inoculation with *P. pneumotropica*. Reddish areas of consolidation extend through most of the upper lobes and part of the lower lobes.

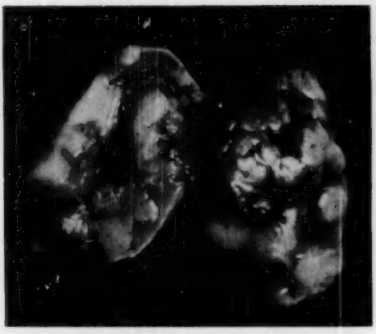

FIG. 2.—Gross appearance of the lung in a mouse surviving for 7 days after inoculation with 6×10^8 organisms of *P. pneumotropica* culture. Firm yellow nodules within areas of resolving greyish consolidation.

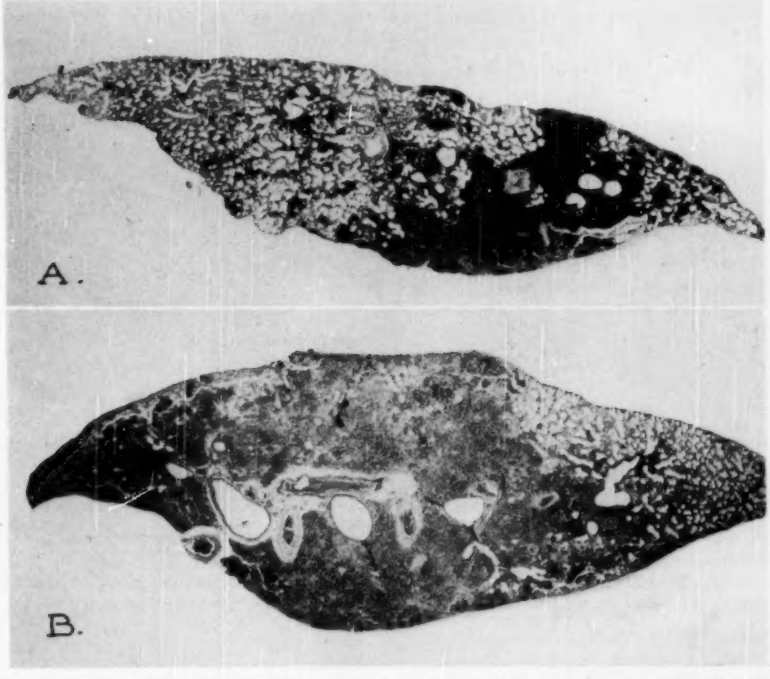

FIG. 3A.—Mouse lung 24 hours after inoculation, showing patchy bronchopneumonia. 9×.
B.—Mouse lung 48 hours after inoculation. The consolidation has progressed, and the areas coalesced, occupying most of the upper lobe. 9×.

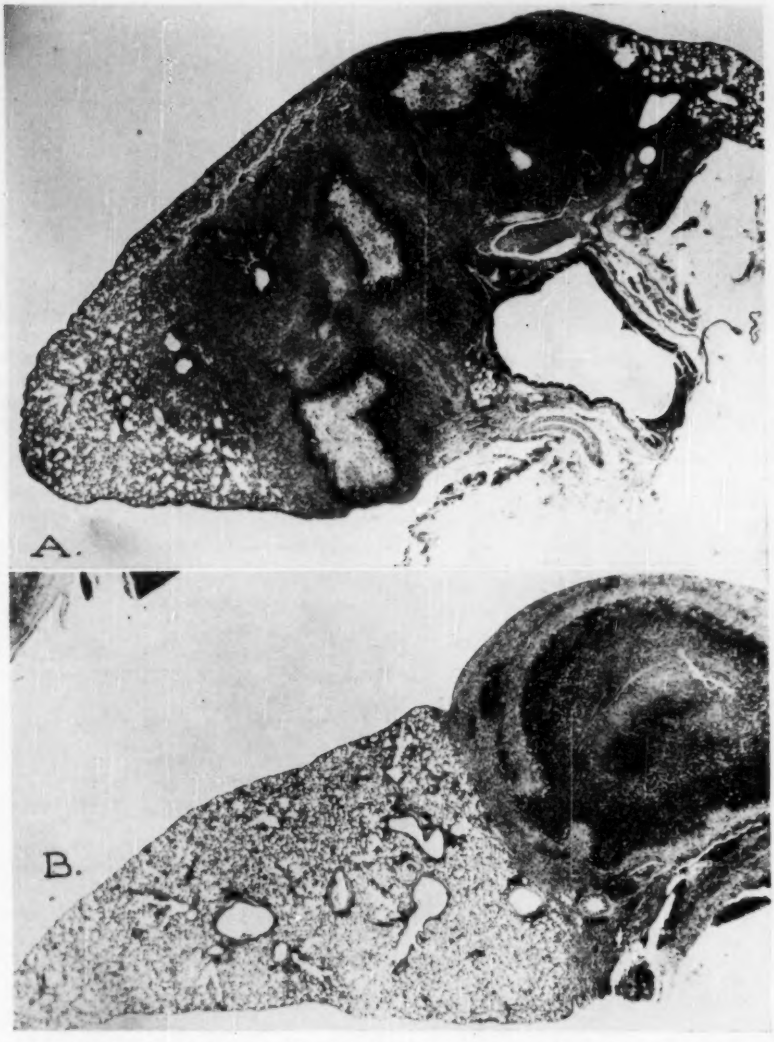

FIG. 4A.—Mouse lung 5 days after inoculation. Definite abscesses have formed within the areas of consolidation. Beginning resolution at the periphery. 14×.

B.—Mouse lung 12 days after inoculation. There is one nodule containing necrotic material and surrounded by a fibrous capsule. The remainder of the lung has resumed its normal appearance. 14×.

Photographed by Mr. Roy Perry.

after infection, when the first definite lesions appeared in the lung, the animals were already quite sick and the organisms distributed widely throughout the body. Peripheral blood cultures were uniformly negative, but heart blood contained many organisms, apparently carried from the lungs, the main focus

TABLE 1.—*Correlation of bacteriological, clinical and gross pathological findings in 21-day-old mice injected intranasally with 6×10^5 virulent* P. pneumotropica.

Sacrificed day after inoculation	Clinical signs (average)	Organ cultures					Gross lesions, lung	Spontaneous deaths
		Lung	Liver	Spleen	Kidney	Heart blood		
1	Drowsy, hunched, chattering, anorexic.	++++	++	++	++	++	Scattered gray.	0
2	Ruffled, cyanotic, lethargic, chattering, some moribund, all dyspneic.	++++	−	−	−	++	Consolidation of up to 4/5 of lung area.	15
3	Dyspnea and cyanosis continue, some improved.	++++	++	+	+	+	Extensive gray consolidation, firmer.	6
4	Less cyanosis, dyspnea, nea, feeding better.	++++	++	++	−	+	Consolidation areas more translucent, same extent.	0
5	Little cyanosis and chattering. Appetite good, fur smooth.	+++	−	−	−	−	Necrotic yellowish centers in gray areas appearing.	0
7	Appearance essentially well. Fur smooth, no dyspnea, gaining weight.	+++	−	−	−	−	Necrotic centers larger, gray consolidation smaller.	1
9	Appearance entirely healthy.	+++	−	−	−	+	Necrotic centers in gray areas same. Spleen enlarged.	
12	Entirely well.	+++	−	−	−	−	Unchanged.	0
19	Entirely well.	++*	−	−	−	−	Unchanged.	0
26	Entirely well.	++*	−	−	−	−	Unchanged except for diminishing size of gray consolidated areas.	0
								0

No pasteurella colonies present, −; 1 to 5 colonies, +; 5 to 20 colonies, ++; 20 to 50 colonies, +++; more than 50 colonies per standard segment of blood agar plate, ++++.
* Lung culture negative, gray areas negative, but necrotic nodule positive.

of proliferation of the pasteurella. On subsequent days the pulmonary lesions progressed to necrosis, and large numbers of organisms were found in them; but the clinical signs of surviving animals gradually subsided, and no bacteria could be recovered from any organ from any organ except the lung.

The most widespread lesions in all lobes on the second and third day were accompanied by the most severe symptoms and the largest number of fatalities. The drowsiness, lethargy and anorexia were probably manifestations from the very large number of bacilli multiplying in the lungs. Serial hematologic examination of 20 mice showed very marked leucopenia indicated by the examples in table 2, perhaps suggesting toxic depression. The dyspnea and cyanosis were apparently a function of the greatly diminished air space of the lungs and the passage of much blood through unaerated lung tissue. The loud "'chattering" has been attributed to abnormality in the lower respiratory tract.[3] Rhinitis or otitis media were encountered frequently by Nelson[3] in "infectious catarrh of mice," where a pasteurella-like bacillus was found in addition to the "cocco-bacilliform bodies" resembling pleuropneumonia organ-

3. Nelson, J. B. 1937, J. Exper. Med. **65**: 833–861.

TABLE 2.—*Hematologic findings in mice infected with* P. pneumotropica.

Mouse no.	Age (days)	Hours after inoculation	Total white cell count	Microhematocrit reading*	Clinical appearance
1	45	2	12,600		Well
		24	6,800		Dyspneic, not toxic
		44	4,200		Very dyspneic, cyanotic
		60	1,000	56	Morbiund (dead at 72 hours)
2	20	2	15,300		Well
		24	5,000		Apathetic, ruffled, dyspneic
		44	2,600		Cyanotic, dyspneic
		60	850	51	Moribund
3	21	6	9,800		Apparently well
		44	3,500		Dyspneic, cyanotic
		60	700	52	Very toxic, almost moribund

* Normal values 45–57.

isms. No such changes occurred in the disease due to *P. pneumotropica*.

At the time when necrotic areas coalesced to form the larger, visible nodules, much of the consolidation throughout the lung tissue had resolved. This probably accounted to a considerable extent for the marked clinical improvement of survivors and disappearance of cyanosis. Pasteurella continued to be present in the involved lung tissue but became less and less abundant until about three weeks after inoculation the uninvolved lung tissue was entirely free. At that time bacilli could be recovered only from the necrotic nodules which were walled off by fibrous tissue. Both the bacteriological findings and the lack of lesions anywhere except in the lungs strongly suggests that *P. pneumotropica* is able to proliferate actively chiefly in lung tissue. From there the organisms appear to be carried passively by the circulation to other organs, being usually rapidly eliminated without ever establishing themselves.

TABLE 3.—*Bacteriological autopsy findings in streptomycin-treated and untreated mature mice infected intranasally with* P. pneumotropica.

Sacrificed day after inoculation	Untreated mice					Treated mice				
	Organ cultures				Deaths	Organ cultures				Death
	Lungs	Liver	Spleen	Heart Blood		Lungs	Liver	Spleen	Heart Blood	
1	++++ ++++ +++	+ ++ +	− − +	+ + ++	0	++++ ++++ ++++	+ + +	+ ++ −	+ + +	0
2	++++ ++++ +++	+++ ++ −	+ + +	+ ++ +	3	++++ − −	− − −	− − −	− − −	0
3	++++ ++ +++	− − −	− − −	− − −	4	− − ++++	− − −	− − −	− − +++	0
4	− ++++ ++++	− − −	− − +	− +++ +++	0	− + −	− − −	− − −	− − −	1
5	++++ − ++++	+ − −	− − −	+++ − +++	4	− − −	− − −	− − −	− − −	0
6	++ ++++ +++	− − −	− − −	− + −	2	+++ − ++	− − −	− − −	− − −	1
7	+ +++ ++	+ − −	− − −	− − −	2	++++ +++ −	− − −	− − −	− − −	3
8					0					4
9					0					2
10					0					3
11–18					2					3
					Total 17					Total 17

Seventy-six 34-day-old mice infected intransally with 10^8 organisms. Half of the mice treated daily with 50 mg/kg streptomycin for 7 days, starting 18 hours after infection. Three mice of each group killed on each of the first 7 days of observation period.
Symbols as in table 1.

The preceding bacteriological and pathological observations were carried out on immature, 21-day-old mice. They were compared to findings in older much more resistant mice, shown in table 3. Histological and bacteriological examination revealed an almost identical pattern of gross lesions and distribution of bacteria, but the deaths were spread out over a longer period, with more than 50% of all fatalities occur-

ring after the 4th day of illness. With the more chronic disease the lesions were more widespread, and the necrosis frequently involved entire lobes or a whole lung.

A similar group of mature mice was infected with the same inoculum and treated with one daily injection of 50 mg/kg streptomycin for 7 days beginning 18 hours after inoculation. Bacteriological studies in this group of animals were performed on 3 mice per day, 20 hours after the last streptomycin treatment. The effect of this subcurative dose of the antibiotic is seen in table 3. It suppressed the spread of organisms from the lung focus, and in a significant number of animals altogether inhibited growth for some time. After treatment was discontinued, however, the mice died as in the control group, the total number of deaths being equal in both series. Thus, no significantly increased resistance resulted from an infection which was held in check by chemotherapy for 7 days.

TABLE 4.—*Method of transmission of* P. pneumotropica *from mother to young. Bacteriological autopsies of female mice during pregnancy and after delivery and of the offspring at intervals after birth.*

Mother sacrificed on day	Culture of							
	Lungs	Liver	Spleen	Heart blood	Breasts	Embryos		
Ca. 2 days before delivery	+	–	–	–	–	–		
Ca. 4 days before delivery	++	–	–	–	–	–		
Ca. 8 days before delivery	+	–	–	–	–	–		
Ca. 10–14 days before delivery	+	–	–	–	–	–		
						Young (composite)		
						Lungs	Liver + spleen	Heart blood
2 days after delivery	+	–	–	–	–	+	+	–
3 days after delivery	+	–	–	–	–	+	+	–
5 days after delivery	++	–	–	–	–	+	–	–
12 days after delivery	+	–	–	–	–	++	–	–
18 days after delivery	+	–	–	–	–	++	–	–

Symbols as in table 1.

Natural transmission of P. pneumotropica

A number of pregnant female mice were sacrificed at arbitrary intervals before the expected litter date, the organs ground and cultured, and the embryos made into a suspension with broth and inoculated into liquid and solid mediums. Table 4 gives 4 instances of healthy pregnant females carrying the pasteurella in their respiratory tract. Neither in the breast, in any internal organ, nor in the embryos could the bacteria be found. In a group of 5 females after delivery, the pasteurella was again present only in the respiratory tract of the mother (table 4), while in the offspring organisms could be recovered from all lung pools and in a few instances also from pooled and ground liver and spleen. The failure to recover pasteurella from embryos of any age is taken to indicate that infection does not take place in utero. The absence of organisms from breasts and milk makes transmission by that route unlikely. The fact that these mothers harbored latent organisms in their respiratory tract and pasteurella were uniformly found in the lungs of the very young offspring strongly suggests that transmission from mother to offspring took place via the respiratory route within less than 48 hours after birth. Many similar experiments confirmed this impression.

The transmission of *P. pneumotropica* by the respiratory route raised the question whether virulent organisms could be similarly transmitted under natural conditions. To test this possibility, contact experiments were carried out.

Groups of mice were assembled in large cages so as to produce rather crowded conditions. A portion of these mice was infected with virulent *P. pneumotropica* culture and the group quired in natural transmission compared with the large dose necessary in successful artificial inoculation. The shortness of effective contact and the markedly lowered susceptibility of pasteurella-free mice, discussed below, are among the many possible conditions interfering with any epidemic spread of disease under these conditions.

Results of streptomycin treatment.—It has been pointed out[4,5] that the pasteurella is highly streptomycin-sensitive and that the antibiotic was effective as a prophylactic or curative agent. The established response of the infection to chemotherapy suggested the use of streptomycin for the establishment of a pasteurella-free mouse stock. Animals free from the latent infection could provide information as to the nature of the marked resistance of stock mice to *P. pneumotropica* infection. If the resistance were due to immunization by subclinical infection in early life, then mice reared in an environment free from pasteurella should be more susceptible. If, on the other hand, the lack of susceptibility should be on an innate, hereditary basis, it should not be affected by the absence of latent infection.

TABLE 5.—*Contact experiments with virulent* P. pneumotropica.

Experiment	Groups making up assembled population	Death/total	Lesion/total	Positive culture/total*
I	20 adult mice carrying latent pasteurella	0/20	1/20	8/20
	20 immature (20-day) mice with latent pasteurella	0/20	0/20	12/20
	10 immature infected intranasally with 10^4 organisms	7/10	1/3	9/10
	20 immature (20-day) pasteurella-free	0/20	0/20	15/20
II	20 immature infected intranasally with 10^4 organisms	16/20	1/4	18/20
	20 immature pasteurella-free	0/20	0/20	13/20

* Lungs cultured at death or at end of three weeks' observations period.

observed for evidence of illness or death. At the end of three weeks all animals were sacrificed and autopsied. Typical experiments are given in table 5. Of 10 artificially infected animals, 7 died 2 to 5 days after inoculation. While the period of active contact was short it was apparently sufficient to effect transfer of organisms to the respiratory tract of immature mice which had been raised free from pasteurella. There resulted no death and only one lesion in all the contact animals. In this experiment, however, the pasteurella-free mice could have acquired their infection from other carriers rather than the mice infected with virulent bacteria. In the next experiment, therefore, only two groups were placed together, one from pasteurella-free stock, the other actively infected with virulent organisms. Again the contact was effective in transmitting pasteurella, but no disease resulted. The simplest explanation for this failure of epidemic spread is the presumably small dose of bacteria ac-

Groups of pregnant mice from the regular colony were brought to the laboratory. Half of each group was

4. Jawetz, E. 1948, Proc. Soc. Exper. Biol. & Med. **68**: 46–48.
5. Jawetz, E. 1948, Proc. Soc. Exper. Biol. & Med. **69**: 105.

started on streptomycin treatment and the offspring raised in isolation. Frequent bacteriological examinations indicated that untreated offspring of treated mothers were always free from pasteurella. Litters from both groups were matched carefully for age, weight, and number of siblings, and challenged with virulent *P. pneumotropica* culture at 18 to 21 days of age. The essential results of two series of such experiments are presented in table 6. Contrary to all expectations, the mortality was significantly higher among animals carrying latent pasteurella. The offspring of streptomycin-treated mothers, although having presumably the same genetic make-up, were definitely more resistant to the infection than parallel litters of untreated mothers. This type of experiment was repeated a number of times always with essentially the same results. It is therefore considered unlikely that some poorly understood factor of chance could be responsible.

TABLE 6.—*Relative susceptibility to virulent* P. pneumotropica *infection of immature mice raised in contact with or free from pasteurella.*

20-day-old mice	Series I Challenge dose		Series II Challenge dose		Totals
	10^8 bacteria	10^5 bacteria	10^8 bacteria	10^5 bacteria	
Pasteurella-free					
Deaths/total	15/29	9/37	9/20	8/30	41/116
Percent deaths	51.7	24.3	45.0	26.6	35.3
Pasteurella-infected					
Deaths/total	29/31	20/30	7/7	7/12	63/80
Percent deaths	93.5	66.6	100.0	58.3	78.7

These results raised the question whether superinfection with very small doses of pasteurella might decrease the resistance to virulent infection. Several groups of immature mice carrying latent pasteurella were given either sterile broth or dilute culture intranasally on the 16th day of life and challenged with 10^6 virulent organisms 10 days later. The results shown in table 7 seem to indicate that multiple exposure to P. *pneumotropica* by the natural portal of entry diminishes rather than increases resistance to subsequent massive infection. This appears to be in agreement with the lower resistance to challenge infection of pasteurella carriers compared to pasteurella-free mice.

The very large numbers of virulent *P. pneumotropica* necessary to produce disease even in immature mice suggest that the native resistance to this infection must be high. This native resistance is apparently a nonspecific mechanism, for it does not interfere with the persistence of considerable numbers of these organisms in the respiratory tract in a latent form. It was of interest to determine whether the production of active immunity might alter this host-parasite relationship by stimulating mechanisms specifically directed against the parasite.

Half of a group of 80 mice 14 days old, and carrying the latent pasteurella, were injected subcutaneously with 10^5 virulent organisms, a dose previously shown to produce satisfactory immunity to challenge infection.[2] Ten days later all 80 mice were sacrificed and their lungs cultured individually. From the 40 nonimmunized mice the pasteurella

TABLE 7.—*Effect of superinfection with sublethal doses of* P. pneumotropica *on resistance to challenge infection.*

Preliminary inoculum given to immature mice (carriers) intranasally 10 days prior to challenge with *P. pneumotropica*	Results of challenge infection with 10^8 virulent *P. pneumotropica* intranasally			
	Series I	Series II	Total	Percent deaths
Sterile broth	10/30*	8/19	18/49	36.7
10^2 organisms of virulent pasteurella culture	17/30	11/16	28/46	60.8

* Deaths/total.

was recovered 26 times, from the immunized group only 7 times (table 8). Thus, it seems that parenteral immunization with a single dose of virulent bacteria may induce sufficient specific response to rid the respiratory tract of latent organisms. A survey of survivors of intranasal infection gave indefinite results. However, early streptomycin treatment of an otherwise lethal infection seemed to interfere with development of a specific immunity mechanism because the pasteurella could be recovered from significantly higher numbers of animals (table 8).

TABLE 8.—*Apparent effect of active immunity on carrier rate of* P. pneumotropica.

Mice	Number of mice	Number of positive cultures from ground lung	Pasteurella carrier (percent of all animals)
24-day-old, received 0.2 ml sterile broth S.C. at 14 days	40	26	65.0
24-day-old, received 0.2 ml 10^{-1} dilution broth culture S.C. at 14 days, ca. 3×10^6 organisms I.C.	40	7	17.5
34 to 42-day-old survivors of I.N. infection with 10^6 virulent organisms	21	9	42.8
35-day-old, treated with 0.2 g/kg streptomycin in 3 doses, afer I.N. infection with 10^6 organisms, then kept in contact with latent pasteurella-infected animals	43	29	67.4

S.C.—subcutaneously.
I.C.—intracutaneously.
I.N.—intranasally.

DISCUSSION

Pathological study of the experimentally induced disease due to *P. pneumotropica* fully confirmed the unusual pneumotropism of the organism.[5] Lesions were, with a single exception, limited to the lungs, and the bacilli were evidently unable to establish themselves in any other organ when carried there passively by the blood stream. This contrast of susceptibility of the respiratory tract and resistance of other organs is a noteworthy phenomenon, rare among bacteria but rather common with viruses.

Intranasal instillation of *P. pneumotropica* into Swiss mice results essentially in a picture of peribronchial consolidation with spreading and coalescence of the involved areas. The reaction is at its height on the second and third days after inoculation, and then there is gradual regression and localization to well defined areas in the upper lobes. At the same time, beginning about the third day, there is necrosis of the exudate with formation of small abscesses. The necrotic areas persist, fuse and become well demarcated with eventual formation of a fibrous capsule. It is probable that most of the initially involved lung tissue undergoes resolution, with evidence of recent infection remaining in the form of increased numbers of polymorphonuclear leukocytes in the interstitial tissue.

It is evident that this is not a specific reaction for any type of organism, many bacteria provoking a similar pathological picture in mice.[6] The moderate splenic enlargement appearing on the 7th day is a common, nonspecific host reaction.

The pneumotropism of *P. pneumotropica* appears to be a feature of the host as much as of the parasite. The pathological picture observed in presumably normal mice inoculated with this bacterium resembles quite strikingly that seen in mice partially immune to *Pasteurella pestis* and infected with large virulent doses of that organism. There, likewise, the lesions are almost exclusively limited to the lungs, while all

6. Dingle, J. H. 1941, Biology of the Laboratory Mouse, Philadelphia, The Blakiston Co. pp. 380–474.

other organs appear entirely normal.[1] The similarity of the two conditions strongly suggests that partial host immunity might also be a factor in the localization of lesions due to *P. pneumotropica*.

P. pneumotropica is latently present in many colonies of laboratory animals, transmitted apparently by the respiratory route from mother to young. The question presented itself whether this high resistance was a function of subclinical infection or an innate, hereditary characteristic of the heterozygous mouse stock used for the experiments. The well established higher susceptibility of the young and the increasingly greater resistance to infection with advancing age would be compatible with gradually developing specific immunity as a result of subclinical infection. On the other hand, superinfection by the respiratory route decreased resistance and militated against the concept of immunization by latent infection.

The striking results of streptomycin chemotherapy offered a useful approach to the problem of hereditary versus acquired immunity in this disease. Removal of the pasteurella by streptomycin treatment from half of a group of pregnant females made it possible to raise mice comparable in every respect except for the presence of latent infection in half of the litters. Surprisingly, the mice kept free from latent infection actually were more resistant to disease and death from superinfection with *P. pneumotropica* than the latently infected group. The difference in susceptibility between the two groups of mice was statistically significant.

Any attempt to explain this curious phenomenon must be based on the fact that double infection of the respiratory tract with *P. pneumotropica* was a prerequisite for its increased pathogenicity. Pre-existing latent infection in the respiratory tract not only failed to stimulate immunity but actually increased susceptibility to superinfection. The original infection with *P. pneumotropica* did not induce significant tissue reaction or clinical illness. Since the tissue response to repeated infection was markedly altered, "sensitization" in its broadest sense may be made responsible although the actual mechanism remains obscure. Parenteral injection of live pasteurella did not produce such "sensitization" but stimulated immunity mechanisms, inducing resistance to superinfection and favoring the removal of *P. pneumotropica* from the respiratory tract.

No conclusive experiments were performed to ascertain whether carriers of *P. pneumotropica* are also more susceptible to other infections than pasteurella-free mice. If, however, the increased susceptibility is a specific phenomenon, it must be considered that contact with the organism leads to a depression in immunity mechanisms. This might then be an instance of the "immunological paralysis" suggested by Felton.[7] Even in very young mice the resistance to *P. pneumotropica* pneumonia is relatively high. More than 10^4 bacteria must reach the lungs before lesions can be observed. This resistance is evidently not induced by subclinical infection but by a hereditary characteristic not interfering with the carrier state and the survival of the parasite in the respiratory tract. When, in such animals, specific immune responses are evoked (through the parenteral injection of bacterial suspensions), *P. pneumotropica* often disappears from the respiratory tract. Thus the relative insusceptibility of even the young mice to challenge infection is due to nonspecific rather than acquired specific factors.

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7. Felton, L. D. 1949, J. Immunol. **61**: 107.

SUMMARY

1. The pathological changes in *Pasteurella pneumotropica* infection are described. The organisms produce lesions only in the lungs following intranasal inoculation. There is a bronchopneumonia progressing to necrosis of the exudate and formation of abscesses. The latter persist and become encapsulated while the remainder of the consolidated areas resolve and regain their normal appearance.

2. The most severe illness and the highest number of deaths occurred on the second and third day after inoculation when the largest areas of lung tissue were involved and unaerated. Very marked leukopenia indicated toxic depression. Necrotic nodules persisted in the lungs for many weeks, without any evidence of illness.

3. The organism appears to spread from mother to offspring by the respiratory route. The natural spread is not of sufficient magnitude to ever result in spontaneous illness. Streptomycin treatment permits elimination of *P. pneumotropica* from a mouse stock kept in isolation. The offspring of pasteurella-free mice are definitely less susceptible to superinfection with *P. pneumotropica* than the offspring carrying the organism.

4. The presence of latent *P. pneumotropica* in the respiratory tract increases susceptibility to superinfection by the intranasal route, while parenteral injection of similar bacterial suspensions decreases it. Possible explanations for for this observation are discussed. The relative resistance of young mice to *P. pneumotropica* infection is not due to subclinical immunization but rather to nonspecific hereditary factors.

FIELD TRIAL OF *SHIGELLA FLEXNERI* III VACCINE. III. COPROANTIBODY STUDIES

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The presence of agglutinins in feces was first described by Davies.[1] Extracts of stools from patients with acute or chronic bacillary dysentery were found to contain specific agglutinins for the causative organisms. Russian investigators[2–4] reported the usefulness of a test for these agglutinins in the diagnosis of dysentery, pointing out that they appeared on the second or third day of illness. They were able to demonstrate these agglutinins in the feces of 60 to 70% of all patients studied. Similar findings were reported more recently by Harrison[5] in the study of shigella and salmonella infections.

The properties of fecal agglutinins and their relationship to immunity were studied extensively by Burrows.[6,7] Evidence was presented indicating the antibody nature of fecal agglutinins; the term "coproantibody" was employed to designate such agglutinins. In immunized guinea pigs and human volunteers, the excretion of coproantibody was found to be independent of the level of serum antibody. Fecal agglutinins reached a peak and disappeared much sooner than circulating antibodies. Coproantibody excretion was shown to be of significance in immunity to experimental cholera infections in the guinea pig. If previously immunized animals were challenged during the period of excretion of coproantibody, an abortive infection was produced. Infection was unaltered when animals were challenged after the disappearance of stool agglutinins, despite the presence of relatively high levels of circulating antibodies.

The United States Navy trial of vaccination for the control of shipboard epidemics of shigellosis furnished an

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opportunity to study coproantibody excretion in a large number of human subjects and possibly to evaluate its significance in immunity to this disease. The background and plan of the program has been described[8] and results of agglutination studies on serums obtained from the subjects reported.[9]

MATERIALS AND METHODS

Stool specimens were obtained from subjects who had received one of the following vaccines:

1. Parenteral shigella vaccine.—This consisted of a heat-killed suspension of *Shigella flexneri* III containing 2.5 billion organisms per ml and was given in three weekly dosages of 0.5, 0.5, and 1.0 ml.

2. Oral shigella vaccine—This consisted of enteric-coated tablets containing approximately 20.0 billion heat-killed *Shig. flexneri* III organisms per tablet. Subjects received one tablet daily for 15 days. Most of these individuals also received three weekly injections of typhoid vaccine as a placebo.

3. Control inoculations.—Subjects in this group received varying amounts of typhoid vaccine which contained 2.0 billion heat-killed *Salmonella typhi* organisms per ml.

Preparation of specimens.—Within 4 hours after collection, stool specimens were placed in approximately 5 volumes of 0.85% saline containing 0.5% formalin and were thoroughly mixed in a Kahn shaking machine for 30 minutes. After standing at 4 C for at least 24 hours they were centrifuged at 3500 r.p.m. for 45 minutes, and the ratio of supernatant solution to stool solids was recorded for each specimen. The supernatant extracts were then decanted and stored at −76 C. When thawed all specimens were centrifuged at 4500 r.p.m. for 30 minutes, and those that failed to clear completely were discarded. A period of 6 to 10 months elapsed between the collection of specimens and the performance of the agglutination tests. Approximately 35% of the extracts were not used because of their failure to clear sufficiently to make reading of the test possible. A total of 2444 specimens was tested.

Agglutination tests.—A modification of the method of Burrows[10] was used. Each specimen was diluted with 0.85% saline to produce a ratio of stool solids to saline extract of 1:25. To tubes containing serial dilutions of the fecal extract (1:25, 1:50, 1:100, etc.) an equal volume of antigen was added and the mixtures were incubated at 37 C for 18 hours. A control containing no antigen was prepared for each extract at a dilution of 1:50. All tests were read by a single observer, using a direct beam of light against a dark background. A positive test consisted of a bead-like, fine, granular clumping in the swirl of antigen after shaking. All tests in which particulate matter was demonstrable in the stool control were eliminated from the study. Specimens showing an original titer of 1:50 or greater were retested, and when positive results could not be obtained, they were recorded as less than 1:25.

Antigens.—Antigens were prepared from the organisms used in the vaccines *Shig. flexneri* III (Be) and *S. typhi* (901-W). The method of preparation of these antigens has been described.[9] A very dilute antigen was used; its light transmission was 99% in the Coleman Jr. spectrophotometer using a #660 filter.

Mouse-protection tests.—Stool extracts were concentrated as follows: The material was dialyzed against distilled water for 48 hours, lyophilized, and reconstituted in 0.85% saline. The following concentrated extracts were tested.

1. One- to two-day postinoculation specimens showing agglutinin titers of 1:50 or greater after parenteral vaccination with shigella vaccine. The concentrate represented the equivalent of a 1:1 dilution of stool solids to saline extract. Each animal received 0.2 ml of this material.

2. Postinoculation specimens taken at intervals of 1 to 30 days from subjects vaccinated parenterally with *Shig. flexneri* III; this material was 10 times more concentrated than one. These specimens had negative agglutinin titers.

The method of testing was that using a constant dose of organisms as described by Cooper.[11]

EXPERIMENTAL RESULTS

A minimum titer of 1:50, corresponding to the 1:20 dilution of Burrows,[10]

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11. Cooper, M. L. and Keller, H. M. 1948, Studies in dysentery vaccination. I. The passive mouse protection test. J. Immunol. **58**: 349–356.

TABLE 1.—*Fecal agglutinin titers for* Shig. flexneri *III in 246 preinoculation and 359 postinoculation* specimens from subjects receiving parenteral shigella vaccine.*

Ship	Specimen	Stool dilutions						≥50%
		<25	25	50	100	200	400	
1	Preinoculation	32	5					
	20–21 days	11		2				15.4
	75–77 days	34	4					
2	Preinoculation	60	5					
	1–2 days	58	14	13	9	3	1	26.5
3	Preinoculation	63	6	1				1.4
	16–17 days	41	2	2	1			6.5
	55–56 days	27	2		1			3.3
4	Preinoculation	30	6	1				2.7
	15–16 days	37	2					
	100–101 days	20	2					
5	Preinoculation	33	4					
	30–31 days	36	1	6				14.0
	45–46 days	21	7	1	1			6.7

* Interval measured from date on which the last dose of vaccine was administered.

was designated as "positive." Certain difficulties were encountered in interpreting the results because of the large number of negative tests in all groups studied and of certain positive tests for which no explanation was apparent, i.e., the increase in positive titers in the control group of ship 2 that received neither the shigella nor the typhoid vaccine. *P* values were calculated in comparing the number of negative and positive tests in the pre- and postinoculation groups for each vaccine.[12] A *P* value of 0.05 or less was considered significant.

Effect of parenteral shigella vaccine.—A total of 246 preinoculation and 359 specimens collected at intervals varying from 1 to 101 days after completion of the course of parenteral shigella vaccine was tested. The only significant increase in positive titers occurred in the group of specimens taken one to two days post inoculation; 26.5% of these subjects developed titers of 1:50 or greater ($P<0.001$). Although increases in the percentages of positive titers were found at later intervals, they were not significant. Table 1 summarizes by ship the

12. Goulden, C. H. 1939, Methods of Statistical Analysis. New York, John Wiley and Sons, Inc.

TABLE 2.—*Fecal agglutinin titers for* Shig. flexneri *III in 244 preinoculation and and 382 postinoculation specimens from subjects receiving oral shigella vaccine.*

Ship	Specimen	Stool dilutions						≥50%
		<25	25	50	100	200	400	
1	Preinoculation	37	2					
	20–21 days	17	1					
	75–77 days	47	3	1				2.0
2	Preinoculation	49	5	2				2.9
	1–2 days	63	18	9	3			12.9
3	Preinoculation	57	10					
	16–17 days	52	9					
	55–56 days	21	4					
4	Preinoculation	36	8	2				4.3
	15–16 days	49	6					
	100–101 days	16						
5	Preinoculation	35	1					
	30–31 days	30	1	3				8.8
	45–46 days	24	3	2				4.3

TABLE 3.—*Fecal agglutinin titers for* Shig. flexneri *III in 468 preinoculation and 745 postinoculation specimens from subjects in the control group receiving* S. typhi *vaccine.*

Ship	Specimen	Stool dilutions						≥50%
		<25	25	50	100	200	400	
1	Preinoculation	70	6					
	20–21 days	26	5	1				3.1
	75–77 days	104	11					
2*	Preinoculation	92	11	6				5.4
	1–2 days	122	36	13	2			8.7
3	Preinoculation	114	17	2				1.5
	16–17 days	85	16	1				1.0
	55–56 days	34	9					
4	Preinoculation	62	11	1				1.3
	15–16 days	89	7					
	100–101 days	56	2	2				3.3
5	Preinoculation	66	9	1				1.2
	30–31 days	69	9	6				7.1
	45–46 days	36	1	3				7.5

* *S. typhi* vaccine omitted in ship 2.

results of the tests after the parenteral shigella vaccine.

Effect of oral shigella vaccine.—A total of 244 preinoculation and 382 postinoculation specimens were tested in this group. There was no significant increase in the number of positive titers at any interval after completion of the course of oral vaccine. Table 2 shows the distribution of the titers obtained in this group.

Effect of typhoid vaccine.—No significant increase in the percentage of positive tests for *Shig. flexneri* III agglutinins was observed in 468 preinoculation and 745 postinoculation specimens from subjects given varying doses of typhoid vaccine as a placebo. Table 3 shows the distribution of the titers obtained. It is of interest to note that the highest incidence of positive tests after vaccination (8.7%) was found in the one- to two-day specimens from ship 2. The subjects in this control group were given no vaccine.

Effect of typhoid vaccine upon coproantibody for S. typhi.—Seventy-seven preinoculation and 114 postinoculation specimens from the control group were tested against an antigen of *S. typhi.* At the intervals studied (16–17 and 30–31 days), no significant increase in fecal agglutinins for this organism was demonstrable. Table 4 summarizes this series of tests.

Mouse-protection tests.—No protective effect against *Shig. flexneri* III organisms was found in concentrated pooled stool extracts. The mice were challenged with organisms varying from 4.0 to 11.0 LD_{50} doses.

Comparison of serum and fecal agglutinins.—An attempt was made to correlate the rise observed in fecal agglutinins with rises in serum agglutinins in the same subjects. There was no observable difference, however, in the serum

TABLE 4.—*Fecal agglutinin titers for* S. typhi *O antigen in 77 preinoculation and 114 postinoculation specimens from subjects in the control group, receiving parenteral typhoid vaccine.*

Specimen	Stool dilutions				≥50%
	<25	25	50	100	
Preinoculation (ship 3)*	69	4	4		5.2
16–17 days (ship 3)	69	4	6	2	10.0
30–31 days (ship 5)†	39	3			

* Received three weekly inoculations of 0.3, 0.3, and 0.3 ml of *S. typhi* vaccine.

† Received three weekly inoculations 0.5, 0.5, and 0.5 ml of *S. typhi* vaccine.

antibody responses of the subjects who failed to show any coproantibody rise and those who developed increased titers of fecal agglutinins.

DISCUSSION

The technical difficulties encountered in the test for fecal agglutinins deserve emphasis. The necessity of using an extremely dilute antigen to prevent masking of the results, as well as the confusion that can be introduced by the presence of particulate matter in the extract being tested, make it imperative that only reproducible results be counted. In every case where original readings could not be confirmed on retesting in the present study, the lower titer was recorded.

The only group of specimens in which there was demonstrated a significant increase in positive titers of coproantibody was collected one to two days after completion of a course of shigella vaccine. In a previous report[9] it was shown that the peak of serum antibody response to this vaccine came at 20 to 21 days post inoculation. This observation is compatible with the finding by Burrows[6,7] that the rise and fall of fecal agglutinins takes place before the serum antibody response. A similar precedence of coproantibody over serum levels has been reported by Harrison[5] in patients with dysentery. He found that in this disease there was an early appearance and relatively short period of excretion of fecal agglutinins, which disappeared completely soon after recovery, at a time when serum immune response was maximal. Goodlow et al,[13] however, in a study of 20 human volunteers receiving T.A.B. vaccine, found that the appearance of the maximum fecal antibody response coincided with the appearance of peak serum titers.

These studies justify no further statement concerning the role of coproantibody in human immunity to shigellosis. The significance of serum antibody in this disease as measured by agglutination tests, is, however, also in doubt.[9]

SUMMARY AND CONCLUSIONS

1. Coproantibody excretion was studied in a group of subjects receiving parenteral and oral *Shigella flexneri* III vaccines and parenteral *Salmonella typhi* vaccine.

2. Significant fecal agglutinin titers were demonstrated only in the one- to two-day postinoculation interval from the group of subjects given parenteral shigella vaccine.

3. Mouse protective antibodies could not be demonstrated in pooled, concentrated specimens.

4. It was concluded that in a significant percent of subjects the parenteral shigella vaccine caused the excretion of fecal agglutinins, the appearance of which was early, transitory, and preceded the period during which the serum antibody response was at a maximum.

5. The technical difficulties encountered in the test for fecal agglutinins are discussed.

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MATERNAL DIET AND THE INFLUENCE OF THIAMINE DEFICIENCY ON THEILER'S GD-VII ENCEPHALOMYELITIS IN MICE

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A number of factors other than specific deficiencies, inanition and genetic constitution of host and parasite have been recognized as influencing experiments on the relation of nutrition to resistance or susceptibility to infection.[1] A possibility not previously studied experimentally is that a deficiency of one factor in the diet of the mother may modify the subsequent response of the young to a deficiency of another in nutrition-infection studies. Of the factors which might well be studied in the diet of the mother, folic acid and unidentified factor(s) in liver were regarded as most likely since the addition of these factors to purified rations containing 9 crystalline B vitamins has improved reproduction and lactation.[2–5] The fact that thiamine deficiency results in a prolongation of the incubation period and a decrease in the incidence of paralysis in mice infected with Theiler's encephalomyelitis or Lansing poliomyelitis is well established.[6–8] A comparison of the influence of thiamine deficiency on Theiler's GD-VII encephalomyelitis in young mice from mothers on diets with and without folic acid and liver is the subject of this report.

EXPERIMENTAL

Weanling mice from our stock colony of Webster Swiss mice were kept in individual cages with screened bottoms, except during the mating periods. At 90 days of age, the mice were mated, one female with one male, for two weeks. After this period they were separated. Males received the same food as the females with which they were mated during the mating period and were maintained on the stock ration at other times. The composition of the experimental diets is shown in table 1. Vita-

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TABLE 1.—*Experimental diets.*

M-3	M-4	M-5	Stock	Thiamine-deficient
Sucrose 72.2% Casein (Smaco) 19.0% Salts IV* 4.0% Corn oil 5.0%	M-3 plus 2% LLP†	M-3 minus folic acid	Purina dog chow	M-3 minus thiamine

Vitamin mixture in mg/100 g (M-3, basal)	
Thiamine hydrochloride	0.3
Riboflavin	0.3
Pyridoxine hydrochloride	0.3
Nicotinic acid	0.5
Calcium pantothenate	2.0
Inositol	100.
Sodium *p*-aminobenzoate	100.
Choline chloride	300.
Biotin	0.01
Folic acid	0.025

* Salts IV mixture used is the following modification of Phillips and Hart mixture (1935, J. Biol. Chem. 109: 657): $CaCO_3$, 1200 g; K_2HPO_4, 1290; $CaHPO_4$, 248g; $MgSO_4 \cdot 7H_2O$, 408 g; ferric citrate, 110 g; NaCl, 670 g; KI, 3.2 g; $MnSO_4 \cdot 4H_2O$, 20.0 g; $ZnSO_4$, 1.0 g; $CuSO_4 \cdot 5H_2O$, 1.2 g.
† LLP—Lederle's Liver Powder, Lot #1432.

mins A and D were administered in the form of oleum percomorphum diluted 1:4 with corn oil, 2 drops per mouse per week. Food and water were available to the mice at all times.

Our experience indicates that folic acid and a factor or factors present in liver improve the ability of the mother to rear her young, although they have no apparent effect on fertility or litter size. Weanling mice on Ration M-3 containing folic acid matured at a normal rate, but females fed this ration after weaning and through gestation and lactation lost weight and were able to rear only 50 to 60% of large litters. With smaller litters these effects were less severe. The addition of liver, Ration M-4, allowed the mother to gain weight during lactation and resulted in an increase in the proportion of mice reared, while the withdrawal of folic acid, M-5, had an adverse effect even on mothers with small litters. Females which received folic acid during one pregnancy were much less severely affected by the absence of folic acid during a subsequent pregnancy.

TABLE 2.—*Theiler's GD-VII encephalomyelitis in mice on thiamine-deficient and optimum diets.*

Diet of female parents	Series	Diet of progeny: Optimum (M-3 basal) vi	Optimum (M-3 basal) f	Thiamine deficient vi	Thiamine deficient f
M-3	109	8/13	0/13	0/11	11/11
	112	15/18	3/18	2/13	9/13
	114	12/13	1/13	0/10	6/10
M-4	109	14/17	0/17	2/18	16/18
	112	11/14	3/14	0/11	6/11
	114	5/6	1/6	0/7	7/7
M-5	109	3/9	1/9	0/9	9/9
	112	6/9	3/9	0/9	9/9
	114	12/14	2/14	0/14	14/14
Stock	112	11/12	1/12	3/12	9/12
	114	19/21	2/21	3/24	17/24

vi—mice showing signs characteristic of GD-VII infection.
f—mice dying without signs characteristic of GD-VII infection.

Following these preliminary observations, the influence of the presence or absence of folic acid and liver in the diet of the mother on the response of the young to thiamine deficiency and infection with GD-VII virus was investigated. Four groups of female mice were fed the four rations, M-3, M-4, M-5 and Purina Dog Chow Checkers (stock), respectively. Litters were obtained three times from each female, and at each weaning time the young from each group were divided into two groups. One group was placed on ration M-3 (control), and the other group was placed on the thiamine-deficient ration. When the thiamine-deficient group had begun to develop signs of the deficiency, both the control and the thiamine-deficient animals were inoculated intracerebrally with 0.03 ml of a 10^{-3} saline suspension of the brain and cord infected with Theiler's GD-VII virus.

The results (table 2) are similar to those observed in corresponding experiments using our regular stock animals maintained on thiamine deficient and on optimum diets.[5] In the deficient animals a decreased incidence of apparent virus infection (paralysis, encephalitis, etc.), a prolonged incubation period and an increased incidence of fatalities in mice showing only the deficiency were observed, whereas short incubation periods, a high incidence of typical signs of infection and a low incidence of mice without signs of infection were observed in the controls. Thus, the presence or absence of folic acid or of liver in the diets of female mice does not influence the course of infection with Theiler's GD-VII virus in their thiamine-deficient progeny apart from the alteration caused by the thiamine deficiency per se.

SUMMARY

No differences were observed in the effect of thiamine deficiency on the resistance of young mice to Theiler's GD-VII encephalomyelitis virus when the mothers were fed diets deficient in liver or folic acid.